Macrophage chemotaxis to apoptotic cells

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Declaration

This is to certify that :

i) *the thesis comprises only my original work towards the PhD*

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Lucy Truman
8th August 2005

In memory of

Marjorie and Desmond Truman

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Abstract

While much is known about the macrophage receptors important for the phagocytosis of apoptotic cells, little is known about how macrophages first "sense" and then move toward dying cells. Work presented here investigates the chemoattractant molecules released by dying cells in the "Come-get-me" stage of apoptotic cell clearance.

The CD14 knockout mouse (CD14-/-) was observed to have increased numbers of free apoptotic cells in the thymus. It was hypothesised that this was due to a defect in macrophage clearance of dying cells. *In vitro* assays revealed that macrophages from the CD14-/- mouse had a partial defect in the phagocytosis of apoptotic cells. Clearance also involves macrophage movement towards the apoptotic corpse. Therefore, it was hypothesised that, in addition to a phagocytosis defect, CD14-/- had impaired chemotaxis to apoptotic cells.

An *in vitro* transmigration assay was developed using the Burkitt's lymphoma (BL) cells as a source of apoptotic cells. In this assay, monocytes and macrophages, but not neutrophils, migrated preferentially to apoptotic BL, and chemotaxis correlated to phosphatidylserine exposure on apoptotic cells. Chemotaxis was abolished when BL were transfected with the anti-apoptotic protein *bcl-2*. Although CD14 was up regulated on the surface of the migrating macrophages, experiments with knockout cells revealed that chemotaxis did not require either CD14 or the scavenger receptor CD36.

Experiments using a viral chemokine antagonist (vMIPII) suggested that fractalkine (CX3CL1) was a candidate "come-get-me" signal released from dying cells. Fractalkine was expressed by apoptotic cells and in chemotaxis assays, CX3CL1 competitively inhibited macrophage migration to apoptotic cells but not to CCL5 (RANTES). Monoclonal antibodies that blocked the chemokine domain of CX3CL1 also inhibited macrophage chemotaxis to apoptotic cells.

This is the first demonstration of the involvement of a known chemokine, CX3CL1 in the clearance of apoptotic cells. In this novel process, cells dying by apoptosis release fractalkine that recruits macrophages primed to efficiently engulf the cell corpse.

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Abbreviations

A

ABCA1	ATP-Binding Cassette Transporter A-1
ACAMPS	apoptotic cell-associated molecular patterns
ADAM	a disintegrin and metalloproteinase
ADP	adenosine diphosphate
Ala	alanine
AMP	adenosine monophosphate
ApoE	apolipoprotein E
APS	ammonium persulphate
ATP	adenosine triphosphate
AxV	annexin V

B

β2 GPI	β2 glycoprotein 1
BAFF	B-cell activating factor of the TNF family
Bcl-2	B cell lymphoma –2
BCR	B cell receptor
BL	Burkitt's lymphoma
BLC	B lymphocyte chemoattractant
BLyS	B lymphocyte stimulator
BMDM	bone marrow-derived macrophages
BRAK	breast and kidney expressed chemokine
BSA	bovine serum albumin

C

C	compliment protein
CAM	cell adhesion molecule
cAMP	cyclic AMP-dependant protein kinase
CCL5	RANTES
CD	cluster of differentiation
CED	cell death defective
CMV	cytomegalovirus

CRP	C-reactive protein
CTL	cytotoxic lymphocytes
Cy5	cy-chrome 5
CX3CL1	fractalkine
CX3CR1	fractalkine receptor

D

d	distilled
DA	dalton
DAB	Diaminobenzidinetetrahydrchloride
DAPI	4',6'-diamine-2'-phenylindole dihydrochloride
DC	dendritic cell
DMEM	Dulbeco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dUTP	

E

EAE	experimental autoimmune encephalitis
EBV	Epstein Barr virus
EBNA-2	EBV nuclear antigen-2
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid-disodium salt
EGF	epidermal growth factor
ELR	glutamic acid, leucine and arginine tripeptide sequence
EMAP II	endothelial monocyte activating polypeptide II
EST	expressed sequence tag

F

Fab	Fragment of antigen binding
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
fMLP	N-formylated-methionine-leucine-arginine

G

GAS6	growth arrest specific gene 6
GDP	guanine diphosphate
GEF	guanine-exchange-factor
Gln	glutamine
GPCR	G protein-coupled receptor
GTP	guanine triphosphate

H

H&E	Haematoxylin and Eosin
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfionic acid)
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HUVEC	human umbilical endothelial cells

I

I	isoleucine
Ile	isoleucine
ICAM	intercellular cell adhesion molecule
IFNγ	interferon γ
Ig	immunoglobulin
IL	interleukin
IMDM	Iscoves modified Dulbecco's medium
IP-10	interferon-gamma inducible protein -10

K

KSHV	Karposi's sarcoma herpes virus

L

LMP	latent membrane protein
LDL	low density lipoproteins
LFA-1	lymphocyte function-associated antigen-1
Log	logarithm
LPC	lysophosphatidylcholine
LPS	lipopolysaccharide
LTA	lipoteichoic acid

Lys	lysine
M	
M	molar
M	methionine
M1	"classically"-activated macrophage
M2	"alternatively"-activated macrophage
MACS	magnetic cell sorting
M-CSF	macrophage colony-stimulating factor
MCP-1	macrophage chemoattractive protein -1
Mer	a membrane tyrosine kinase
MFG-E8	milk fat globulin protein EGF factor 8/lactadherin
MHC	major histocompatability complex
MIP-1α	macrophage inflammatory protein-1α
MM6	mono mac 6
MMP9	matrix-metalloproteinase 9
mRNA	messenger ribonucleic acid
N	
NFκB	nuclear factor κB
NGS	normal goat serum
NK	natural killer (cell)
O	
Ox-LDL	oxidised-low density lipoprotein
P	
PAF	platelet activating factor
PAGE	polyacrylamide gel elecrophoresis
PAMPS	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PE	phycoerythrin

PECAM	platelet endothelial cell adhesion molecule
PGE2	prostaglandin E2
PI	phosphatidylinositol
PI	propidium iodide
PI3K	phosphatidylinositol 3 kinase
PLA2	phospholipase A2
PLT	paucity of lymph node T cells
PMA	phorbol 12-myristate 13-acetate
PPP	platelet poor plasma
PRR	pattern-recognition-receptors
PS	phosphatidylserine
PSR	phosphatidylserine receptor
PTX	pertussis toxin

R

RANTES	regulated in activation, normal T cell expressed and secreted
RGD	Arginine, Glycine and Aspartate
RPE	R-phycoerythrin
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute
RNA	ribonucleic acid
RNA-S19	RNA protein dimer S19
RS	aminoacyl-tRNA synthetase
RSV	respiratory syncitial virus
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction

S

SAP	serum amyloid P
SHPS-1	Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1
SR	scavenger receptor
SR-PSOX	scavenger receptor of PS and oxidated phospholipids
SD	standard deviation

SEM	standard error of the mean
SCM-1 β	single cell motif-1 β
SIP-Rα	sphingosine 1 phosphate receptor α
SLC	secondary lymphoid chemokine
SLE	systemic lupus erythematosus
SUPP	supplemented with

T

T	threonine
TACE	tumour necrosis factor-α-converting enzyme
TAM	tumour associated macrophages
TBS	Tris-buffered saline
TdT	terminal deoxynucleotidyl transferase
TEM	transmission electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	transforming growth factor-β
TH1	CD4+ T helper cell type 1
TH2	CD4+ T helper cell type 2
TLR	toll-like-receptor
Tris	Trishydroxymethyl-methylamine
tRNA	transfer RNA
TNFα	tumour necrosis factor-α
TNFR	tumour necrosis factor receptor
TSP-1	thrombospondin-1
TUNEL	deoxynucleotide transferase-mediated dUTP nick end labelling
Tyr	tyrosine

U

UK	United Kingdom
USA	United States of America
UVB	ultraviolet light B

V

V	valine

vMIP	viral macrophage inflammatory protein
V-region	variable region
Val	valine
VEGF	vascular endothelial growth factor

W

WBC	white blood cells

Chapter 1

Introduction

1.1 Introduction

In order to ensure their timely removal, it is known that apoptotic cells release chemoattractive molecules that attract phagocytes. This thesis will investigate the of role macrophage receptors in the chemotaxis to, and engulfment of apoptotic cells.

1.2 Apoptosis

When a cell dies it usually goes quietly, by a process of programmed cell suicide called apoptosis. But when it is faced by overwhelming infection or damage, the cell may simply explode in an un-planned pattern of death (Cotran, 1989). Accidental death is called necrosis, it happens without warning and cells may have limited time to signal their distress. First the cell swells, and then it bursts releasing organelles like mitochondria, proteolytic granules and lysosomes. Cellular debris can sometimes be detected in healthy tissues and this can be due to "secondary" or late necrosis. Unlike apoptotic cell contents, the organelles released during necrosis are not wrapped in a cover of plasma membrane (Savill, 2002).

Critically, throughout apoptosis, the plasma membrane appears to remain intact and cell contents do not leak out and attract the attention of the immune system. The name "apoptosis" is coined from the Greek meaning "leaves falling off the trees," and was first described by Kerr, Wyllie and Currie in 1972 (Kerr, 1972). It is a physiological process that regulates cell numbers and maintains homeostasis. During apoptosis, the cell carefully packages up its nuclear material and safely recycles intracellular constituents. This is done in a step-wise manner and the accompanying morphological changes can be monitored microscopically. First the lipids in the plasma membrane are rearranged. Best known of the apoptotic signals is the translocation of the phospholipid, phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane (Fadok, 1992); This rearrangement is coordinated by scramblase and translocase enzymes (Williamson, 2002). The exposure of PS can be measured by the calcium-dependent binding of AnnexinV (Koopman, 1994). Next, the cytoskeleton collapses and consequently the cell shrinks, the nuclear envelope is dismantled and the DNA becomes fragmented. The different lengths of DNA resulting from this enzymatic process give a typical

"ladder" pattern when the DNA is run out on a gel. Small blebs appear on the plasma membrane often containing nuclear material and sometimes, autoantigens (Casciola-Rosen, 1994).

Apoptosis is essential for the remodelling of tissues, for example, the dissolution of the inter-digital finger webs during embryogenesis. It is also important during lymphocyte development and in response to infection. Germinal-centres are sites of intense B cell proliferation and programmed cell death, and here, apoptotic cells are seen frequently, alongside the "tingible body" macrophages that are busy engulfing them. Likewise, apoptotic cells are found in the thymus and bone marrow, two sites where self-reactive clones are deleted. In the periphery, apoptosis is important in the control of viral and intracellular infections. The proteases and nucleases that are activated during apoptosis also destroy viral DNA and so limit the spread of infection. Therefore, apoptosis is a safer method of disposing of cells than necrosis. It is not surprising that cytotoxic T lymphocytes (CTL) can initiate programmed cell death through the extrinsic pathway.

A number of extracellular signals (extrinsic) and intracellular (intrinsic) pathways can trigger apoptosis (Figure 1.1). CTL expresses Fas ligand that binds the Fas (CD95) death receptor on target cells (Trauth, 1989). Fas is a member of the Tumour Necrosis Factor receptor family (TNFR) and is an example of how the extrinsic pathway is triggered. Upon CTL induction, the receptor clusters at the plasma membrane and recruits adaptor proteins able to trigger an enzyme cascade resulting in cell death. The proteases that are responsible for the disassembly of the cell are called caspases, "C" because they have cysteine at their active site and "asp" because they cleave proteins at specific aspartic acid residues (Nicholson, 1977; Nicholson, 1999;Cohen, 1997). Caspases are not synthesized *de* novo, but lie in pro-enzyme form "waiting" for the pro-apoptotic signals. Each caspase sequentially activates another in the chain, and so the number of active protease enzymes is amplified at each step of the process. The intracellular pathway is often recruited during the activation of the extrinsic pathway. p53, the most commonly mutated gene in human malignancies, induces apoptosis in damaged cells in order to prevent the replication of faulty genes (Lowe, 1993). Under the control of genes targeted by p53,

mitochondria release cytochrome C. This activates caspase 3, the executor enzyme, which is the common to both pathways.

Caspases are closely regulated by a set of genes in the Bcl-2 (B cell lymphoma) family (Chao, 1998). Bcl-2 inhibits apoptosis by blocking the release of cytochrome c (Wang, 1993). Bcl-2 is a 24kDa membrane protein that was cloned in 1986 (Cleary, 1986). Cell lines used in the present work have been transfected with Bcl-2 in order to prevent apoptosis (Henderson, 1991;Milner, 1992). Two other members, Bax and Bak also stimulate cytochrome c release, whereas Bad, is pro-apoptotic because it binds and inactivates bcl-2. Another family member, Bid is able to insert the lipid lysophosphatidylcholine (LPC) in to the mitochondrial membranes and this membrane perturbation promotes the leakage of cytochrome c (Goonesinghe, 2004). Interestingly, LPC can also be released from apoptotic cells and is chemoattractive to monocytes (Lauber, 2003).

Many of the phases of apoptosis can be viewed under a microscope, except from very early on in the apoptotic programme when dying cells may be indistinguishable from their live neighbours. However, new techniques have been developed to help spot these fresh corpses (Leers, 2002). Even so, apoptotic cells are vanishingly rare except for at the lymphoid sites mentioned previously. The reason for their scarcity is that they are swiftly removed by either neighbouring cells or by the "professional phagocytes", the macrophages. The process of engulfment of apoptotic cells is the last, but sometimes forgotten stage of the apoptotic process *in situ* (Fadok, 1999).

1.3 Clearance of apoptotic cells

Apoptotic cells are rarely seen *in vivo* because their clearance is so quick and efficient. Over 100 years ago, Elias Metchnikoff first described macrophages phagocytosing dying cells (Karnovsky, 1981;Shafrir, 1995). When apoptotic cells are seen in histological tissue sections, they are nearly always associated with a macrophage (Kerr, 1972). Therefore, macrophages have been named the "professional" phagocytes. It is likely that the macrophage's main role is to remove apoptotic cells from areas with high death rate, as happens during inflammation or development (reviewed in Gregory, 2004). Although not studied as deeply, non-

specialised neighbouring cells also have a role in the clearance of cell corpses (Parnaik, 2000). Removal of dead cells by these "amateur" phagocytes may account for the majority of the day-to-day clearance required to keep up with the pace of physiological cell turnover. Amateur phagocytes, like retinal epithelium and mesenchymal cells are less mobile than macrophages and so on-the-spot phagocytosis proceeds more slowly in these cells (Marmorstein, 1998; Wood, 2000). Local removal of dead cells is very efficient. The PU1 knockout mice have no macrophages and yet develop normally because of the work of these amateur phagocytes (McKercher, 1996). The nematode worm, Caenorhabditis elegans (C.elegans), also has no macrophages and uses unspecialised neighbours to remove apoptotic cells efficiently (Wu, 1998).

Caenorhabditis elegans

The study of the worm *C.elegans* revealed a link between the genes mediating apoptotic cell-clearance and those that signalled death. When a cell dies by programmed cell death it has the foresight to activate undertaker genes that are required for the removal of its cell corpse. These seven ced (Cell Death Defective) genes are there to allow development to proceed smoothly or more simply, in order to recycle cell parts. Mutations in the ced genes leads to the persistence of 131 "extra" cells. None of the ced genes is essential because the worm can tolerate the presence of these persistent apoptotic cells (Devitt, 2004 66;Ellis, 1991). However, 600 million years of evolution has insured that even primitive organisms have developed mechanisms to remove dying cells swiftly and quietly. The more complex fruit-fly, *Drosophila melanogaster*, cannot survive without apoptosis and has developed haemocytes that are able to remove dying cells in order for development to proceed normally (Franc, 1996). Haemocytes may be the forerunners of mammalian macrophages, the so-called "professional" phagocytes.

All seven ced-genes have human homologs with a conserved function in both the clearance of apoptotic cells and in cell migration (Yuan, 1993; Hengartner, 1994). The ced genes are grouped into two separate cassettes and function as two pathways that possibly interact. The first pathway contains the genes (CED1, CED6 and CED7) and their human homologs (*LRP/CD91, hCed6/GULP and ABCA1)* respectively (Liu, 1998;Wu, 1998;Zhou, 2001). Ced1, like CD91 is a transmembrane

receptor for apoptotic cells and Ced 6 is an adaptor protein that aids clustering of this receptor (Su, 2002). Indeed, CD91 also forms clusters when interacting with apoptotic cells (Ogden, 2001). Ced 7 facilitates the distribution of lipids on both the macrophage and the apoptotic-cell membranes. The human homolog, the ABCA1 transporter also alters the local composition of lipids in the plasma membranes. ABCA1 is required to be on both the apoptotic cell and the phagocyte for engulfment to occur (Wang, 2003;Williamson, 2002). By moving phosphatidylserine, ABCA1 affects the overall fluidity of the plasma membranes of both cells and increases cell mobility and so enhances clearance (Hamon, 2000;Hamon, 2002).

The second pathway or cassette contains four genes (CED2, CED5, CED12 and CED10) and their human homologs (*crkII, DOCK 180, ELMO and Rac/GTPase* respectively), (Wu, 1998; Zhou, 2001; Reddien, 2000). These are genes important for both cell mobility and chemotaxis (Wu, 2001). The group consists of an adaptor protein (ced2/crkII), a protein GEF (guanine-exchange-factor) complex. Together ced5 and ced6 remove GDP that allows GTP to bind to ced10/Rac (Brugnera, 2002). The human homolog of ced 10, is the Rho-family-member Rac, that promotes the extension of the actin cytoskeleton. This is important for both the outgrowth of pseudopodia for engulfment and also for macrophage migration through tissues towards apoptotic cells. The upstream receptor for the second pathway is not known but it could be either an integrin-like receptor or perhaps a chemokine receptor (Albert, 2000).

Removal of apoptotic cells

A brief glance at the number of receptors implicated in apoptotic cell-clearance in Figure 1.2, confirms that mammals are far more complicated than worms. Even so, the question of why so many molecules are needed for engulfment cannot be avoided. Suggestions of receptor redundancy have been put forward to explain these vast numbers, reasoning that engulfment of apoptotic cells is a critical process that needs fail-safe back up molecules. In support of a redundancy theory, some knockouts (c1q-/- and MFG-E8-/-) show very specific defects that are limited to a single organ for example in the MFG-E8-/- mouse apoptotic cells accumulate in germinal centres (Hanayama, 2004); And others, for example, CD14-/-, display no adverse effects to the persistence of apoptotic cells (Devitt, 2004). Undoubtedly,

clearance is important, but perhaps this view has been overstated particularly as many of the receptors like C1q and CD14 have other immunological jobs to do. It is likely that different strains of mice and different tissues have a preferred method of clearance. In addition, these molecules do the clearing up together, as a team. A model has been proposed where some molecules simply anchor the apoptotic cell to the phagocyte e.g. CD14 (Devitt, 2004) and others signal for engulfment e.g. MFG-E8 (Hanayama, 2004). This has been described as "tethering" and "tickling (Henson, 2001; Hoffmann, 2001). This may be too simplistic because clearance of apoptotic cells can be divided into at least six phases: "Come-get-me", "eat-me", "don't-eat-me", "tether", "tickle" and finally the immunological "consequences" of engulfment. With such complexity, one or two examples, pertinent to the work of this thesis will be chosen to illustrate each of the five steps.

"Come-get-me"

Lysophosphatidylcholine, S19, EMAPII and fractalkine

A cell that is in the process of committing suicide will release chemoattractive molecules that advertise its presence to phagocytic cells. Lysophosphatidylcholine, is an example of a chemoattractive molecule released by apoptotic cells (Lauber, 2003) and will be discussed in Section 1.6. If neighbouring cells within are within range, these "come-get-me" signals need not travel too far and may help direct tissue macrophages towards the target cell. The longer-range signals, such as the chemokines released by endothelial cells, like RANTES (CCL5), are able to recruit monocytes from the circulation into organs. The role of fractalkine (CX3CL1) in macrophage chemotaxis to apoptotic cells will be discussed separately in Section 1.5 (Bazan, 1997).

Macrophage receptors for apoptotic cells

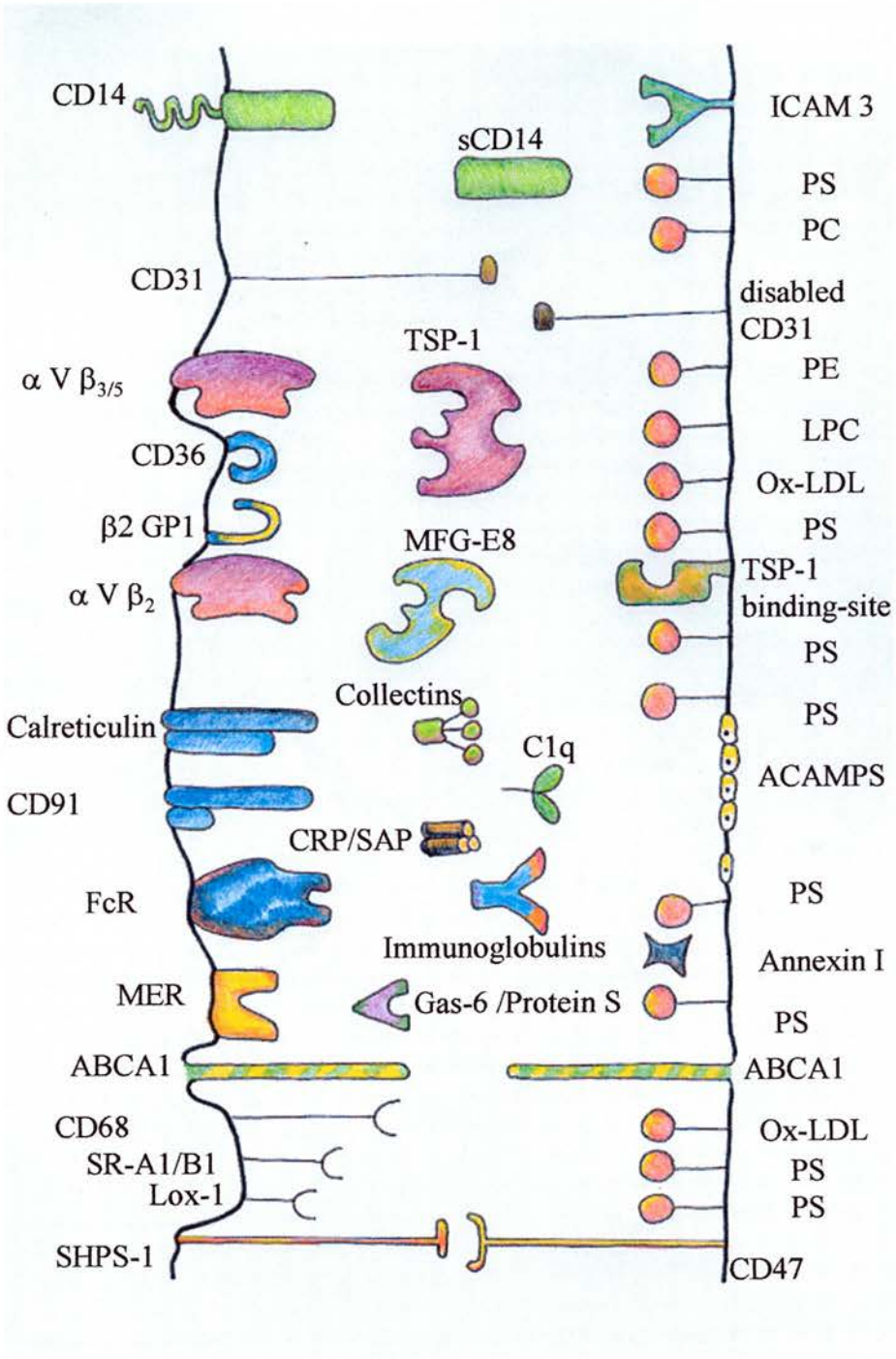


Figure 1.2

"Eat-me"

Phosphatidylserine

"Eat-me" and "don't eat me" signals require the phagocyte and the apoptotic cell to be in close proximity. Phosphatidylserine (PS) is the most famous of all the "eat-me" signals (Fadok, 1992). PS normally resides on the inner leaflet of the plasma membrane but during apoptosis this asymmetry is lost and membrane lipids become redistributed through the actions of two enzymes a flipase and a scramblase (Bratton, 1993; Williamson, 2002). The detailed mechanisms that control PS redistribution are not fully understood to date. But PS is one of the earliest steps in apoptosis and precedes morphological events like cell-shrinkage and bleb formation.

FITC-labelled annexin V, that binds to surface PS in the presence of calcium is commonly used for detecting apoptotic cells by flow cytometry (Koopman, 1994). During apoptosis the dying cell transports a related annexin, annexin 1 from the cytoplasm to the external leaflet of the plasma membrane alongside PS (Arur, 2003). If antibodies or RNA silencing inhibits the translocation of annexin 1, this prevents engulfment of the apoptotic cell. Annexin 1 has a role in both tethering and engulfment. It is unknown whether annexin 1 binds directly to a phagocytic receptor. The fMLP (formylated Methionine-Leucine-Phenylalanine) chemokine receptor expressed on monocytes but especially on neutrophils can bind annexin 1 (Walther, 2000). The fMLP receptor binds to N-formylated peptides produced by bacteria and guides leukocytes to sites of infection.

Alternatively, annexin 1 may not bind to a receptor but attach directly to PS on the apoptotic cell surface. As for annexin V, this binding is calcium dependent. Early on in apoptosis there is a spike in intracellular calcium levels that triggers the mobilisation of annexin 1 from the cytoplasm to the plasma membrane. When faced with the lower calcium concentration of the extracellular space, annexin tends to aggregate. This may pull PS and other apoptotic cell-ligands into clusters that can be recognised by the C1q-calreticulin-CD91 complex (Ogden, 2001).

A receptor for phosphatidylserine

No macrophage surface receptor had been shown to bind directly to PS until 2000 when Fadok and colleagues described the phosphatidylserine receptor (PSR) (Fadok, 2000). However, doubts about the identity of the PSR have recently come to light (Williamson, 2004). PSR was discovered through screening a panel of monoclonal antibodies that could block the uptake of apoptotic cells. The same unbiased strategy was used when CD14 was found to be a receptor for apoptotic cells (Devitt, 1998). The monoclonal antibody 217G8E9 blocked engulfment and stimulated the release of TGFβ (Fadok, 1998). Using phage display, the peptide recognised by mab 217G8E9 was sequenced and cloned. Later studies showed that the *psr*-gene product was a nuclear protein, and it seemed unlikely that it was involved in apoptotic cell clearance at the periphery of the cell (Cui, 2004).

Two mouse PSR knockouts on the mixed 129 (agouti brown mouse)/C57BL/6 (black mouse) backgrounds were produced (Li, 2003; Kunisaki, 2004). Both of these psr-/- reported perinatal lethality and a failure of apoptotic cell-clearance. In particular, the lungs of the PSR-/- embryos were abnormal. Lungs develop by extension and branching, not by involution and apoptosis, as occurs during mammary development. It again seemed unlikely that a failure to engulf apoptotic cells was driving this phenotype. Around the same time the worm homolog of the psr, the upstream receptor for pathway two, was knocked out in *C.elegans*. Again, there was persistence of apoptotic cells but unexpectedly, the mutation was less severe than deletions of other ced genes (Wang, 2003).

A third group made a psr-/- on the C57BL/6 background and again this mouse had gross embryological malformations, but importantly, there was no defect in the removal of apoptotic cells (Böse, 2004). Furthermore, the antibody, mab217G8E9 bound to both wild type and psr-/- cells. It seems that the discovery of the psr gene was a case of mistaken identity and that mab217G8E9 may cross react with another surface protein. The spectrum of persistence of apoptotic cells shown across the knockouts from different backgrounds is a warning to interpret differences between mouse strains very carefully (Mills, 2000; Weitzman, 2004).

"Don't-eat-me"

CD31 and CD47

Recently it has been shown that live cells express "don't eat me" signals to prevent them being engulfed by macrophages. A homotypic interaction between CD31 / (PECAM) allows the important discrimination of live from apoptotic cells. CD31 normally repels CD31 on a phagocyte, or more correctly, it signals the macrophage to "go-away" (Brown, 2002). However, during apoptosis, CD31 is somehow disabled and the CD31's signal is no longer "go away". How CD31 becomes altered is unknown, it may involve changes in the carbohydrate moieties as may happen to ICAM3, perhaps before being recognised by CD14 (Moffatt, 1999; Gregory, 2000; Gregory, 1998). Red blood cells escape destruction in the spleen by expressing CD47 that signals "don't-eat-me" to splenic macrophages expressing SIP-Rα. (Oldenberg, 2000). Signals from live cells may be more important *in vivo* where clearance is a swift and early event. If a macrophage recognises an altered apoptotic ligand, tethering and engulfment of the apoptotic cell will ensue.

"Tether"

CD14

The lipopolysaccharide (LPS) -receptor CD14 is an example of a protein that tethers apoptotic cells (Devitt, 1998; Devitt, 2004). Indeed, soluble CD14 that is abundant in plasma can discriminate between live and apoptotic cells by opsonising LPS-like motifs (Devitt, 2004). Similar to CD14, other important immunoregulatory proteins also bind to apoptotic cells. This group of well-characterised proteins including antibodies and complement proteins have been shown to opsonise apoptotic cells (Ogden et al 2001). IgM binds lysophosphatidylcholine on the surface of dead cells, and auto-antibodies from patients with Systemic Lupus Erythematosus (SLE) were shown to bind to the surface blebs of apoptotic cells (Casciola-Rosen, 1994). These opsonins can bridge the gap between the apoptotic cell and phagocyte by binding Fc and complement receptors on macrophages (Hart, 2004).

Apoptotic cells are often recognised by pattern-recognition-receptors (PRR) like CD14 and collectins (Vandivier, 2002; Gregory, 2000; Ogden, 2001). PRR are conserved germ line-encoded receptors that recognise repetitive molecular patterns on pathogens. One example of a pathogen-associated-molecular pattern (PAMP) is lipopolysaccharide (LPS). PAMPS are essential for pathogen survival and the PRR's that recognise them have evolved over millions of years. In fact the PRR "Toll" genes are conserved between plants and humans (Medzhitov, 1997). This has led to some of the "altered" PAMP-like ligands found on apoptotic cells to be called, perhaps indiscriminately, ACAMPS (Apoptotic-cell-associated-molecular patterns, (reviewed in Gregory, 2004).

Although many molecules like CD36, complement and CD68, can recognise apoptotic cells, but there is very little evidence these bind to PS directly (Kodama, 1996; Ogden, 2001; Savill, 1992; Ren, 1995). The vitronectin receptor ($\alpha v\beta 3$), the integrin receptor $\alpha v\beta 5$ and MER all employ bridging molecules that first opsonise apoptotic cells before gathering them in (Scott, 2001). The importance of CD14 in macrophage interaction with apoptotic cells is discussed in more detail in a later section of this chapter.

"Tickle"

MFG-E8 and CD36

MFG-E8 (Milk fat globule epidermal growth factor protein 8) is an example of a bridge that connects PS to $\alpha v\beta 3$ and $\alpha v\beta 5$ (Hanayama, 2002). As its name suggests, it is found secreted in breast milk. It binds to PS via a clotting factor VIII-homology domain and it has an RGD (Arginine, Glycine and Aspartate) motif at its amino-terminus that enables it to bind to integrins. MFG-E8 is expressed by "tingible body" macrophages in germinal centres, where it is important in the receptor mediated-phagocytosis of apoptotic cells. MFG-E8 knockout macrophages tethered apoptotic cells but could not engulf or "tickle" them (Hanayama, 2004). In addition these knockout mice have enlarged spleen, but the cell ratios are normal. This bulk is not simply due to the persistence of apoptotic cells but may be related to MFG-E8's effect on the lymphocytes life span. Alternatively MFG-E8 may affect B and T cell recruitment and retention in the spleen.

CD36 is another phagocyte receptor that uses integrin receptors to engulf apoptotic cells (Savill, 1992). It is a well-characterised class B scavenger receptor for oxidised low-density lipoprotein that also recognises altered lipid on the surface of apoptotic cells (Ren, 1995). Scavenger receptors are mainly expressed on macrophages and bind negatively-charged, acetylated and oxidised low density lipoproteins (LDL) (Krieger, 1994; Chang, 1999). Like wise, CD36 is expressed by macrophages and platelets and recognises apoptotic cells in a complex of thrombospondin and αvβ3 (Savill, 1992).

Monoclonal antibodies raised against LDL on apoptotic cells inhibited macrophage clearance, whereas over-expression of CD36 in a non- phagocytic cell line allowed the phagocytosis of apoptotic cells. Only "altered" or oxidised LDL (but not native LDL) and anti-CD36 antibodies could inhibit uptake of apoptotic cells (Ren, 1995). Although CD36 will bind to PS, it binds to the apoptotic-cell indirectly through a thrombospondin bridge, that links to an unknown ligand. Whether or not it binds PS directly, CD36 is required for the PS dependent uptake of apoptotic cells (Fadok, 1998). Recently, it was discovered that CD36, like TLR2, binds Lipoteichoic acid (LTA) and diacylglycerides, both PAMPs displayed by Gram-positive cells (Hoebe, 2005). Antibodies against PAMPS like LPS and LTA have been used to identify novel apoptotic cell-associated patterns (Tennant, 2004).

CD36 appears to have a choice of which integrin receptor to bind, either αVβ3 or αVβ5. CD36 interacts with αVβ3 on macrophages to engulf apoptotic cells quietly, but when CD36 associated with αVβ5 on dendritic cells, it can lead to cross presentation of self-antigens. This interaction could potentially result in autoimmunity (Albert, 1998; Kodama, 1996).

The immunological consequences of engulfing apoptotic cells

Autoimmunity and TGF-β

The many different types of receptor implicated in the phagocytosis of apoptotic cells are striking (Figure 1.2a). It is intriguing that such a diverse group of receptors is involved in the phagocytosis of apoptotic cells and yet, despite this variety, the outcome of engulfment is almost always non-inflammatory (reviewed in (Savill, 2002; Henson, 2001; Grimsley, 2003).

The macrophage is not simply a waste disposal and recycling outfit, although the consequences of it engulfing a dying cell may be neutral and environmentally sound (Kurosaka, 2003). The phagosome is a secure place to break-up apoptotic cells and it is said, to prevent the environmental damage caused by necrotic cells. In order to prevent such damage the accepted wisdom is that the apoptotic-cell membrane must be intact before "secondary necrosis" has occurred. However, *in vitro* observations show that macrophages prefer to eat leaky cells. It may not be the mode of cell death, but the type of cell that is dying that is critical (Gregory, 2004). The membranes of both necrotic and apoptotic cells can stimulate the release of TGFβ by macrophages (Fadok, 2001). When a neutrophil dies it is more likely to trigger inflammation than an apoptotic lymphocyte. It may be that necrotic death of short-lived granulocytes early on in the immune response may help to amplify the immune response early. However, a proinflammatory reaction to the death of a lymphocyte (present during the later stage of an infection) might delay healing and even promote chronic inflammation. In addition, elastases released from apoptotic neutrophils could cleave the putative anti-inflammatory PSR, but conceivably pro-inflammatory receptors could be cleaved by the same mechanism (Vandivier, 2002).

Most often, the clearance of apoptotic cells by mononuclear phagocytes results in anti-inflammatory effects, with the production of TGFβ, IL-10 and PGE2 (Voll, 1997; Fadok, 1998; McDonald, 1999). In addition, apoptotic cells can release these mediators themselves (Ogden, 2005; Gao, 1998; Chen, 2001).

However, when apoptotic cells are fed to macrophages in the presence of LPS it must be remembered that the natural cell's response to LPS alone may be to balance the early release of TNFα by a later, perhaps more sustained, production of IL10. This is why it is not surprising to see that apoptotic cells do not completely quench the initial inflammatory response to LPS (Lucas, 2003). The simple binding of apoptotic neutrophils to monocyte produced IL-10 in response to LPS (Byrne, 2002). A cuff of apoptotic cells, that can't be phagocytosed, surrounds macrophages in the spleen of the MFG-E8 -/- mouse. Despite binding apoptotic cells these mice still develop inflammatory autoimmune disease at 40 weeks (Hanayama, 2004;Hanayama, 2002). Other knockouts like C1q and MER and also have autoimmune disease in the presence of apoptotic cells (Botto, 1998; Scott, 2001).

However, apoptotic cells persist in CD14-/- without any inflammatory consequences (Devitt, 2004). One mechanism for the autoimmunity may be the cross-presentation of self-antigens on MHC by dendritic cells (Albert, 1998; Albert, 2004). Autoantibodies against cell membrane-phospholipids can direct pro-inflammatory responses through Fc-receptor signalling (Kim, 2003; Hart, 2004). The interpretation that all necrotic cells are pro-inflammatory whereas apoptotic cells are not, over-simplifies the complex interaction between the dead cell and the phagocyte (Gregory, 2004).

CD14

CD14 is a 55kDa transmembrane receptor that binds the phospholipids PS and PC and also lipopolysaccharide (LPS) from gram-negative bacteria (Jack, 2000; Ulevitch, 1999). CD14 is predominantly expressed on myeloid cells, monocytes and macrophages. CD14 has a short glycosylphosphatidylinositol GPI-linked cytoplasmic tail that contains no signalling elements and therefore, CD14 is thought to pair up with other receptors, like the Toll-Like Receptors (TLRs) and complement receptor, CD11b, in order to signal (Akashi, 1999; Perera, 2001).

CD14's most famous role is that of a receptor for bacterial endotoxin, LPS, where it is responsible for the production of TNFα that can lead to septic shock (Wright, 1990; Beutler, 2001). The discovery that CD14 is also a receptor for apoptotic cells was initially surprising. The importance of CD14, in this context was discovered by screening a panel of "orphan" antibodies against leukocyte surface structures for their capacity to inhibit macrophage and apoptotic cell interactions. The monoclonal antibody 61D3 was shown to block recognition and phagocytosis of apoptotic cells and after transient expression cloning and probing, it was revealed that 61D3 bound to CD14 (Devitt, 1998). What is more, epitope-mapping experiments showed that 61D3 bound close to the LPS binding pocket. In fact this binding was so close that the antibody could block TNFα production after LPS stimulation (Devitt, 1998). Because of the close proximity of 61D3 binding to the LPS binding site, an intriguing question arises: Do apoptotic cells display LPS-like ligands on their surface? If so, it would be predicted that the CD14-mediated clearance of apoptotic cells pro-inflammatory. However, apoptotic cells persist without causing inflammation in the CD14-/- mouse (Devitt, 2004).

In addition to LPS, CD14 binds many other ligands, such lectins and different phospholipids (Yu, 1997; Wang, 1998; Chow, 1999; Poltorak, 2000; Sugiyama, 2001). Therefore Devitt and colleagues investigated whether CD14 was an alternative receptor for PS (Devitt, 2003). CD14 could bind to PS but it had a higher affinity for phosphatidylinositol (PI), phosphatidylcholine (PC) and preferred to bind to phosphatidylethanolamine (PE) most of all. Although PI liposomes were able to block LPS binding they had no effect on the phagocytosis of apoptotic cells (Akashi, 1999). PS liposomes also had no effect on the CD14-dependent clearance of apoptotic cells (Devitt, 2003). These results show that CD14 is not an alternative receptor for the recognition of phosphatidylserine on apoptotic cells. Later experiments showed that CD14 is a tethering molecule and does not have a phagocytic role. Tethering events can be measured in isolation from phagocytosis by lowering the temperature below 20C. At this temperature, macrophages tether but do not phagocytose apoptotic cells (Devitt, 2004).

ICAM-3 (CD50) has been proposed to be an apoptotic ligand that CD14 recognises. ICAM-3 is a highly glycosylated leukocyte marker that may become "alternatively glycosylated" during apoptosis. These apoptotic changes to the carbohydrate structure of ICAM-3 appear to be recognised by CD14. Surface ICAM-3 on live cells normally binds LFA-1 and not CD14. During programmed-cell death, ICAM-3 loses some of its affinity for LFA-1 and seems to bind to CD14 instead (Gregory, 1998; Moffatt, 1999). However, direct binding between ICAM-3 and CD14 has not been demonstrated.

CD14 and Toll-like Receptors (TLR)

TLR4 is a well-described member of the important Toll-like receptor family, which appears to bind to LPS in co-operation with its co-receptor CD14, and signals through NFκB to up-regulate the transcription of pro-inflammatory cytokines like TNFα and chemokines like CXCL8 (IL-8) (Medzhitov, 1997; Chow, 1999; Poltorak, 2000; Ulevitch, 1999). The transfection of a constitutively active toll receptor into the monocyte cell-line THP-1 was able to up-regulate the co-stimulatory molecule CD86 (Medzhitov, 1997). This showed that TLRs formed an important link between the innate and adaptive immune systems. Rather that just being an ancient relic, quite separate from the adaptive response, innate pathogen recognition receptors may

instruct the adaptive system (Medzhitov, 1997; Medzhitov, 2003). Additionally, TLRs may set the "flavour" of the consequent adaptive response, for example TH1 versus TH2 (Medzhitov, 2000; Chow, 1999; Fearon, 1996). Although CD14 binds to TLRs when it has bound to LPS, when CD14 has bound to an apoptotic cell it does not signal through a TLR (Shiratsuchi, 2004). However if CD14 is associated with a necrotic cell it may signal through TLR2 (Li, 2001). Significantly, phagocytosis of apoptotic cells appears not to require TLR signalling (Blander, 2004).

$CD14^{low}$ and $CD14^{high}$ monocyte subsets

The heterogeneous lymphocyte lineage has been divided into subsets each with specific phenotypes and individual functions. There is growing evidence that monocyte subsets also exist (Ziegler-Heitbrock, 1996). Large monocytes were separated from small monocytes be elutriation and were shown to have different phenotypes. Smaller monocytes had higher antigen-presenting ability but produced fewer reactive oxygen species than the larger cells (Ziegler-Heitbrock, 1991). Ziegler-Heitbrock first described $CD14^{high}$ and $CD14^{low}$ human monocyte subsets in 1988 (Ziegler-Heitbrock, 1988). Cells in the $CD14^{high}$ subset are typical monocytes. The minority, $CD14^{low}$-subset, normally represents only 5% of circulating monocytes but numbers can dramatically increase during sepsis and in HIV infection (Blumenstein, 1997; Skrzeczyñska, 2002; Fischer-Smith, 2001; Thieblemont, 1995). These $CD14^{low}$ monocytes also express the FcγIII receptor, CD16. Because the CD14+/CD16+ subset expressed high levels of MHCII molecules, it was thought that these monocytes were in fact, dendritic cells (DC). Phenotypic and functional analysis have shown both similarities and differences between the monocytes and the DCs (Ancuta, 2000; Almeida, 2002; Thomas, 1994). It is likely that both the $CD14^{high}$ and $CD14^{low}$ monocytes have the potential to differentiate into DCs *in vivo* as was found to be the case in the mouse (Geissmann, 2003; Sallusto, 1994). In a reverse-transmigration, *in vitro* model of leukocyte-trafficking across endothelium, CD14+/CD16+ human monocytes were able to develop into migratory dendritic cells (Randolph, 2002).

The exact role of $CD14^{low}$/CD16+ monocytes remains an enigma. There is evidence that $CD14^{low}$/CD16+ monocytes have a "pro-inflammatory" phenotype. $CD14^{low}$/CD16+ monocytes were major producers of TNFα and their numbers were

increased during sepsis (Belge, 2002; Blumenstein, 1997). The $CD14^{high}$ and $CD14^{low}$ human monocytes each expressed different chemokine receptors and had the potential to migrate to different compartments of the body. $CD14^{high}$ monocytes migrated preferentially to CCL2 (MCP-1) *in vitro* compared to the $CD14^{low}$ subset, which expressed higher levels of CCR5 mRNA, and accordingly, preferentially migrated to RANTES (CCL5) and MIP-1α (CCL3) (Weber, 2000). The receptor for fractalkine (CX3CR1) is highly expressed on the $CD14^{low}$ /CD16+ subset and mediates the arrest of these monocytes to HUVEC transfected with fractalkine (CX3CL1) whereas the $CD14^{high}$/CD16- cells responded to CCL2 but not CX3CL1 (Ancuta, 2003). Mice have an equivalent subset to humans that is $CX3CR1^{high}$/CCR2-/Gr1-. Geissmann et al used an elegant adoptive transfer approach to show that $CX3CR1^{high}/CD14^{low}$ monocytes migrated into non-inflammed sites whilst the $CX3CR1^{low}$ subset trafficked to the peritoneum (Geissmann, 2003). This paper suggested that fractalkine receptor could be important in the constitutive migration of monocytes out of the blood into tissue.

In addition to chemokine receptors, $CD14^{high}$ and $CD14^{low}$ human monocytes have different phagocytic receptors. The FcγRIII receptor CD16 on $CD14^{low}$ monocytes has been mentioned, but these monocytes also have increased class A scavenger receptors I/II (ScR-AI/II) on their surface, that controls the types of lipid they bind (Draude, 1999). Interestingly there was no difference in CD36 expression between the subsets. The $CD14^{low}$ macrophages had impaired uptake of acetylated-oxidised LDL but both the $CD14^{high}$ and $CD14^{low}$ monocytes bound oxidised LDL equally well.

There was also differential expression of the glucocorticoid receptor (GCR) between the subsets. The $CD14^{low}$/CD16+ subset disappeared from the circulating monocyte pool after infusion of high-dose glucocorticoids because these GCR-expressing cells were more susceptible to steroid-induced apoptosis (Dayyani, 2003). Steroid treatment is known to promote monocyte maturation into macrophages and to enhance the phagocytosis of apoptotic cells (Heasman, 2003; Giles, 2001). Monocytes have a very low ability to phagocytose apoptotic cells that improves as they mature into macrophages. It is not known whether $CD14^{high}$ and $CD14^{low}$ monocytes mature into specific macrophage subsets. Two macrophage well

described subsets are the "classically-activated/M1" and the "alternatively-activated/M2" macrophages (Gordon, 2003). However macrophages exhibit plasticity and are less fixed in there function for example, compared to T cells. Although $CD14^{low}$/CD16+/ $CX3CR1^{high}$ monocytes have a phenotype more similar to alveolar macrophages than to peritoneal macrophages, it is not known if they precursors for an "alternatively activated" macrophage.

It is unknown whether $CD14^{low}$ monocytes subsequently develop into $CD14^{low}$ macrophages. *In vitro* experiments would predict that $CD14^{low}$ macrophages would be impaired in their ability to phagocytose apoptotic cells and this was the case *in vivo* where a tethering defect led to the accumulation of apoptotic cells in the CD14 deficient mouse (Devitt, 2004). However, paradoxically, the $CD14^{low}$ /CD16+ monocytes produce more TNFα in response to LPS compared to $CD14^{high}$ monocytes (Draude, 1999). This was not due to the absolute levels of CD14, but to an increase in the expression of TLR in the $CD14^{low}$/CD16+ subset. The phenotype of human $CD14^{low}$ macrophages will be investigated in Chapter 3.

Experiments using a CD14 -/- knockout mouse have given a clearer picture of the physiological role of CD14 in the phagocytosis of apoptotic cells (Devitt, 2004). Future experiments using the CD14-/- mouse will show whether CD14 is a functional marker of this small- $CD14^{low}$ /CD16+ monocyte subset or simply an incidentally expressed phenotypic label.

The CD14 knockout mouse (CD14-/-).

The first CD14-/- knockout mouse was created in 1997 on a C57BL/6 background using a targeted gene approach. A frame-shift in the CD14 gene was caused by replacing a 272 base-pairs section within *CD14* with a *neo'* gene. The disrupted gene was then expressed in embryonic stem cells, injected into blastocysts and the resulting mice were bred to homozygosity (Haziot, 1996). CD14-/- animals were fertile, produced normal-sized litters and had no developmental defects.

Naturally, the first studies on the knockout mice concentrated on how the loss of CD14 affected the immune system and defects in the clearance of apoptotic cells were not studied initially. CD14-/- mice were resistant to LPS and could survive up to 10 times the LD_{100} than the wild type, and secreted virtually no TNFα (Haziot, 1996). CD14-/- mice were also resistant to very high inoculations of gram-negative

bacteria. In a model of *Salmonella typhimurium* peritonitis there was some impairment of neutrophil chemotaxis into the peritoneum in the CD14-/- animals but the recruitment of F4/80-positive monocytes was unaffected (Yang, 2002). These results suggested that CD14 was not strictly required for the neutrophil influx, but in the absence of CD14, neutrophil recruitment was delayed and there was no local inflammatory response. Despite a normal ability to phagocytose whole bacteria, the CD14-/- mice were able to tolerate a higher bacteraemia than their wild type littermates and were slower to eliminate infections (Yang, 2002). However, in pathogen-free conditions, CD14-/- mice had a comparable lifespan to wild type controls. The fact that CD14-/- mice bred successfully was of interest, to the Gregory laboratory that was studying how the loss of CD14-/- might affect the clearance of apoptotic cells.

Apoptotic cell clearance in the CD14-/- mouse

Previous work from the Gregory laboratory, showing that CD14 is involved in the clearance of apoptotic cells by human macrophages *in vitro*, has been extended to show that CD14 is also involved in clearance by murine macrophages *in vivo* (Devitt, 1998; Devitt, 2004). CD14 appears to be a tethering molecule for apoptotic cells. Histological examination of the CD14-/- mouse revealed that there were increased numbers of apoptotic cells in the spleen and thymus of the CD14-/- (Devitt, 2004; Gregory, 2002). When the number of apoptotic cells that were in contact with macrophages was compared to the number of non-associated or "free" apoptotic cells; the CD14-/- mouse had a greater number of "free" apoptotic corpses compared to wild type controls. Examination of the finger webs of the developing CD14-/- embryo showed that the macrophages were gorged-full of apoptotic bodies and were qualitatively different to wild type macrophages (Gregory, unpublished observation). This suggested that the clearance of apoptotic cells during development in the CD14-/- mouse was somehow different to the wild type but the outcome was the same, namely, a healthy live birth.

The accumulation of apoptotic cells in multiple tissues of CD14-deficient mice may be the result of a defect in apoptotic-cell removal resulting in the persistence of apoptotic cells that are not associated with phagocytes *in situ.* Both the wild type and the CD14-/- mice had comparable numbers of macrophages and the

rate of apoptosis was also shown to be equal (compared to the wild type) (Devitt, 2004). There are at least two explanations for the accumulation of dying cells in the CD14-/- animals in relation to their interactions with macrophages: Either, phagocytosis of apoptotic cells was impaired in the CD14-/- animals; or, the absence of CD14 caused a chemotactic defect leading to a reduced efficiency in the recruitment of macrophages to apoptotic-cells.

1.4 Chemokines and their receptors

The finding that loss of CD14 resulted in the accumulation of apoptotic cells led to the examination of apoptotic cells as a source of chemoattractant molecules for macrophages. Chemotaxis is the directional movement of a cell up a chemoattractant concentration gradient and is important in a number of biological processes including leukocyte recruitment to sites of inflammation and into tumours, neurogenesis and fibroblast migration during wound healing (reviewed in Zlotnik, 2000; Singer, 1986). Chemotaxis of a circulating leukocyte through the endothelium into an inflammed tissue progresses step-wise through primary adhesion and rolling, activation and arrest, firm adhesion and finally, diapedesis (Springer, 1994; Lasky, 1992; Butcher, 1996; Webb, 1993). A mixture of *in* vitro and condition-of-flow studies has shown that a cell first senses the chemical gradient using 7-transmembrane-spanning, G-protein coupled, chemokine receptors. It next orientates itself along the axis of the concentration gradient (Singer, 1986) and extends pseudopodia from the edge of the cell facing the highest chemokine concentration and retracts its trailing edge away from the lowest concentration (Zigmond, 1974). Next the leukocyte binds weakly to the endothelium through cell-surface lectins called selectins. The adherence is so weak that the force of passing blood is able to propel leukocytes forwards in a rolling motion (Muller, 1999). Once a chemokine gradient has been sensed, cells become activated to bind more firmly through adherence to integrin receptors (Thompson, 2000).

Chemokines

Chemokines are small (8-17kDa), positively charged "chemotactic cytokines" that induce cell migration. The development of EST (expressed sequence tag)

databases that can pick out small proteins from the genome suggests that it is likely that most of the human chemokines have already been discovered (Zlotnik, 2003). To date, the human chemokine-family contains 42 chemokine ligands and 18 chemokine receptors in all. Many other molecules like bacterial fMLP, C5a and platelet-derived lipids can also attract cells but are not strictly classed as chemokines and will be referred to as chemoattractant molecules.

The chemokine superfamily has been divided into four groups and renamed according to the distribution of conserved cysteine residues (C) found at the amino terminal (Zlotnik, 2000). The four groups are: C, CC, CXC and CX3C where X represents an amino acid separating two disulphide bonds. Chemokines are given the suffix "L" to denote ligand (CX3CL1 (fractalkine) for example), and receptors have the suffix "R". Cellular expression of receptors and ligands from these groups broadly represent function. CCR are expressed on monocytes and lymphocytes and CXCL chemokines, like CXCL8 (IL-8) also contain additional ELR (glutamic acid, leucine and arginine) tripeptide sequence that attracts CXCR-expressing neutrophils.

Most of the chemokines fall into either an inflammatory group or a non-inflammatory group usually expressed in a tissue specific manner (Kurth, 2001). The first chemokines to be identified were all pro-inflammatory and had important roles in recruiting leukocytes to sites of inflammation (DeVreis, 1999). Inflammatory chemokines like CXCL8 (IL-8) and CCL2 (MCP-1) are found in gene clusters. These genes may have divided in response to evolutionary pressures, perhaps from viruses (like cytomegalovirus (CMV) and Herpes) that can produce chemokine antagonists. These gene clusters produce chemokines that can bind to more than one receptor. For example, CCL5 (RANTES) binds to three receptors, CCR1, CCR3 and CCR5.

In contrast, homeostatic chemokines, (like CCL21) are found at isolated chromosomal locations and have a monogamous relationship to their receptors. CCL21 is expressed by high endothelial venules and by lymphatic endothelial cells, and its job is to recruit T cells into lymph nodes (Gunn, 1999). Loss of CCL21 function results in the *plt* (paucity of lymph node T cells) mouse, which has grossly abnormal secondary lymphoid organs (Nakano, 1998).

Chemokines can perform other diverse functions in addition to directing cell migration. They can regulate haematopoiesis and angiogenesis (Kunkel, 1999; Chensue, 2001) and many are also antimicrobial peptides. At high concentrations they can lyse bacteria in a similar way to defensins (Chensue, 2001).

CX3CL1 (fractalkine) and CXCL16 (SR-PSOX) are two unusually large chemokines that are also cell adhesion molecules. They both have long mucin stalks that are cleaved to release their N-terminal chemokine domains. Fractalkine and CXCL16 can arrest the cells expressing their cognate receptors (CX3CR1/fractalkine receptor and CXCR6/Bonzo respectively) under flow conditions. Pathogens like malaria, *E.coli, S aureus* respiratory syncitial virus (RSV) and CMV all take advantage of this binding opportunity (Chensue, 2001; Kledal, 1998; Tripp, 2001; Hatabu, 2003). CXCL16 is a scavenger receptor that not only binds to phosphatidylserine but also participates in the phagocytosis of a wide variety of bacteria (Shimaoka, 2003; Minami, 2001). A recent publication has shown that many other chemokines share this scavenger receptor activity and suggests that there is a close evolutionary relationship between chemokines and scavenger receptors like CD36 (Shimaoka, 2004).

1.5 Fractalkine and its receptor

Fractalkine (CX3CL1) has an unusual structure of a "chemokine on a stalk" which is shared by only one other chemokine CXCL16, a member of the CXC group. As discussed above, CXC16 has scavenger receptor activity, binds oxidised phospholipids and is involved in phagocytosis of bacteria (Shimaoka, 2003; Shimaoka, 2004). No similar scavenger activity or phagocytic role for fractalkine has been identified to date. The fractalkine knockout mouse and its receptor knockout (CX3CL1-/- and CX3CR1-/- respectively) had no overt phenotype and there was no mention of an accumulation of apoptotic cells in the organs of these mice (Jung, 2000; Cook, 2001).

CX3CL1 structure and tissue distribution

Fractalkine was discovered as an experimental offshoot from research on the C branch of chemokine genes that contains XCL1 (lymphotactin) and XCL2 (single cell motif-1 β (SCM-1β)). Fractalkine has now been given the systematic name CX3CL1 (Zlotnik, 2000). Bazan talks about why he called the new chemokine fractalkine in the original paper, quoted below.

As closer inspection of one branch of the chemokine superfamily, the C branch, yielded evidence of another, the CX3C, rather as in fractal geometry (where dynamic, self-referential branch patterns in chaotic systems are revealed at increasing scales of magnification), we have dubbed this new CX3C chemokine 'fractalkine' (Bazan, 1997)

Fractalkine is a large (373 amino acid) membrane-bound, chemokine with a unique CXXXC motif originally found in (but not restricted to) non-haemopoietic cells, like neurons and epithelial cells (Pan, 1997; Harrison, 1998; Lucas, 2001). Fractalkine expression on endothelial cells and human umbilical endothelial cells (HUVECs), can be induced by proinflammatory cytokines like TNFα and it is also up-regulated during brain inflammation (Pan, 1997). There was some initial confusion about the tissue distribution of fractalkine until elegant studies by Lucas et al revealed that this was due to an antibody cross-reacting with CD84 (Lucas, 2001). Fractalkine is a gene directly targeted by p53 and so potentially may be expressed by tumours where this promotor is active (Shiraishi, 2000).

CX3CL1 chemokine and adhesion molecule

The 90-100kDa, full-length transmembrane form of fractalkine is an adhesion molecule that is cleaved to release the soluble chemokine domain (Garton, 2001; Tsou, 2001; Hundhausen, 2003). Antibodies to both the N and C-terminals of fractalkine have identified many different-sized bands by western blotting. Pulse chase analysis of cells that were over-expressing fractalkine, showed that CX3CL1 was synthesized as a 50-75 kDa precursor that was further glycosylated to yield the 100kDa mature protein found at the cell surface. The mature 100kDa fractalkine was

then cleaved to release the 85kDa chemokine domain and to leave behind a 20kDa C-terminal stalk on the cell surface (Garton, 2001; Lucas, 2001).

ADAM10 and ADAM17 (a disintegrin and metalloproteinase) are two distinct metalloproteinases that cleave fractalkine (Garton, 2001; Tsou, 2001; Hundhausen, 2003). ADAM10 is a disintegrin–like metalloproteinase that is responsible for the constitutive shedding of fractalkine and ADAM17 (also known as TACE, Tumour necrosis factor-α-Converting Enzyme) was responsible for the induced release of fractalkine after treatment with a phorbol ester (PMA). It is not known how important the individual contributions of ADAM-10 and ADAM17 are to chemokine release *in vivo* but it is likely that they coordinate the release of soluble fractalkine. Dual regulation of fractalkine would direct cell adhesion and local cell trafficking

Full-length, membrane-bound fractalkine binds its receptor via the N-terminal domain. It is the chemokine domain, and not the stalk, that is responsible for firm adhesion of CX3CR1 positive cells under flow conditions. The stalk simply presents the chemokine to its receptor partner in an advantageous aspect (Haskell, 2000). Two basic residues in the chemokine domain of fractalkine are responsible for interaction with the receptor, Lysine-7 and Arginine-47. Replacement of either residue by alanine affected cell-cell adhesion, but only the Arginine-47 position was important for chemokine activity (Harrison, 2001).

Interestingly, firm adhesion of CXCR1-positive cells did not require G-protein signalling and was not inhibited by pertussis toxin (PTX) (Haskell, 1999). However, chemotaxis to soluble fractalkine did require G-protein signalling. Fractalkine signalling through CX3CR1, could boost cell adhesion to ICAM-1 and fibronectin indirectly, by increasing integrin avidity, this time in a PTX-sensitive manner (Goda, 2000). Fractalkine is generally believed to be a weak chemokine that induced a variable chemotactic response in monocytes and monocyte-cell lines. Monocyte chemotaxis to CX3CL1 (fractalkine) was a fraction (5%) of that to CCL2 (MCP-1) (Imai, 1997). Overall, leukocyte-adhesion may be fractalkine's primary function (Umehara, 2001). Gene targeting of fractalkine generated CX3CL1 -/- mice that had a remarkably normal phenotype (Cook, 2001). CX3CL1-/- animals had no reproductive, behavioural or gross histological abnormalities compared to wild type

mice. Fractalkine-deficient mice had a slight decrease in the number of F4/80 circulating monocytes and an increased number of CD4 T cells in the gut epithelium. However, CX3CL1-/- reacted normally to i.p thioglycollate and efficiently cleared away a *Listeria monocytogenes* infection.

Fractalkine receptor

The fractalkine receptor (CX3CR1) is a typical 7-transmembrane G-protein-coupled, chemokine-receptor (Imai, 1997). It is primarily expressed by monocytes and macrophages but not neutrophils (Beck, 2003). Cytotoxic lymphocytes, NK cells, γδ-T cells, and terminally differentiated $CD8^+$ T cells, all expressed CX3CR1 (Nishimura, 2002). IFNγ, but not IL-4 or IL-13, was able to induce the release of soluble fractalkine and CX3CR1 was predominately expressed by TH1-polarised $CD4^+$ cells (Fraticelli, 2001).

Fractalkine-receptor-positive smooth muscle cells responded to fractalkine produced by inflammatory cells within the atherosclerotic plaque (Lucas, 2003). $CX3CR1^+$ smooth muscle cells reinforced the plaque and prevented it rupturing. This reparative role suggested that cells expressing CX3CR1 had a constitutive, non-inflammatory role. The human CD16+ monocyte subsets, which are likely to be equivalent to the mouse $CX3CR1^{high}$ monocytes, were able to migrate across unstimulated endothelium (Randolph, 2002).

CX3CR1 polymorphisms and disease

The fraction of human CD16+ monocytes is increased in the blood of HIV patients and fractalkine was up-regulated in patients with HIV and AIDS-related encephalitis (Tong, 2000; Erichsen, 2003). It is likely that the $CD16^+$ monocytes in this study would have also been $CX3CR1^+$ since $CD16^+$ monocytes preferentially migrate to fractalkine (Ancuta, 2003). As well as being recruited into HIV-infected brain, two isoforms of CX3CR1 were discovered to be co-receptors for the HIV virus (Garin, 2003). Fractalkine receptor also binds to another viral component, a glycoprotein from RSV (Tripp, 2001).

Fractalkine and its receptor have an important role in atherosclerosis, which is the chief cause of ischemic heart disease. V/I249 and T/M280 are two relatively common polymorphisms of the human fractalkine receptor that form the I249/M280

haplotype present in about 28% of the population. The I249 mutation protects carriers from cardiovascular disease independently of smoking and other acquired risk factors (McDermott, 2001; McDermott, 2003; Moatti, 2001). Where as the M280 mutation is associated with an increase in the risk for a brain infarct (Combadiere, 2003). The difference between I249 and M280 in causing monocyte adhesion in these two clinical syndromes is not well understood. It is possible that they separate cell pathways. Alternatively, the M280 polymorphism that was more adherent than I249 in an *in vitro* flow chamber assay, might allow for tight monocyte adherence in the lower-pressure cerebral system, that is not relevant to pathogenesis of arteriosclerosis in the high-pressure vessels

CX3CR1 knockout mice had no obvious phenotype, just like fractalkine knock out mice (Jung, 2000). Monocyte recruitment and DC maturation and migration were normal and microglial accumulation at the site of a facial nerve injury was similar to wild type. The biological function of fractalkine was exposed when Christophe Combadiere crossed CX3CR1-/- with the Apolipoprotein E (apoE) knockout mouse. The apoE mouse is a model used to study vascular disease because these mice succumb early to atherosclerosis. The phenotype of the CX3CR1-/- /apoE -/- double knockout-mice was striking because they had less atherosclerosis (Combadiere, 2003). The absence of fractalkine receptor resulted in far smaller plaques that contained more smooth muscle cells that were less likely to rupture. These results showed that fractalkine played a direct role in atherosclerotic lesion development on this mouse model.

The importance of fractalkine in graft rejection has been studied, because of the high levels of fractalkine-receptor expression on mouse NK cells. Cardiac allografts in CX3CR1-/- mice survived longer than wild type mice treated with a sub-therapeutic dose of cyclosporin (Haskell, 2001).

The CX3CR-/- mouse has not been studied in the context of lymphoma or any other tumour to date but fractalkine receptor has been identified in glioblastoma multiforme (Zlotnik, 2003).

1.6 Macrophage chemotaxis towards apoptotic cells

Cells dying by apoptosis release chemoattractive molecules in order to attract macrophages that will subsequently engulf them (Ravichandran, 2003). The cell surface changes on apoptotic cells that make apoptotic cells more "palatable" to macrophages have been studied in detail but only recently have the chemokines that attract macrophages been investigated. Apoptotic cells release chemokines when the plasma membrane is intact, whereas necrotic cells can leak out many potential chemotactic molecules, like platelet activating factor (PAF) (Bratton, 1993).

Three chemotactic molecules have been described that are released from apoptotic cells: 1) RNA protein dimer S19 (RNA-S19) (Horino et al., 1998), 2) Endothelial Monocyte-Activating Polypeptide II (EMAPII) (Wakasugi, 2003) and 3) lysophosphatidylcholine (LPC) (Lauber, 2003). In addition, blebs, shed from the surface of apoptotic germinal centre B cells were also shown to have chemotactic activity, or were an effective system for distributing the chemotactic molecules on their surface (Segundo, 1999). Interestingly, none of these molecules belonged to the classical chemokine families.

S19 ribosomal protein dimer is a monocyte chemoattractant

S19 is a subunit of the ribosomal protein that was isolated from the synovium of a patient with rheumatoid arthritis and shown to be chemoattractant for monocytes (Nishiura, 1996). A large number of apoptotic cells have been shown to persist in patients with rheumatoid arthritis (Firestein, 1995). During apoptosis, 45kDa S19 homodimers were released that were chemotactic to monocytes but not to neutrophils at a concentration of 10^{-9}M (Horino, 1998). S19 was responsible for the predominantly monocyte infiltration that was found commonly in chronic inflammation (Yamamoto, 2000). A transglutaminase enzyme that was induced during the process of apoptosis catalysed the homodimerisation of S19 between Gln1378 and Lys122. The homodimers did not form in live cells and were specifically released from dying cells. S19 was detected in the supernatants of apoptotic cells 24-30 hours after heat treatment and by 48 hours the activity of S19 was quenched *in vivo* by a serine protease. S19 was released from two different cell

types, an epithelial and a myeloid cell-line (AsPC-1 and HL-60 respectively) suggesting it was a mechanism common to many cells (Nishimura, 2001).

S19 homodimers bound to the G-protein-linked complement receptor for C5a 9Shibuya, 2001). Indeed, S19-dimers competed with C5a for the C5a-receptor binding despite there being only 4% homology between the protein sequences. The dimerisation process seems to have brought together residues critical for interacting with the receptor. The C5a receptor has two binding sites for the attachment of either the S19-dimer or C5a, the first pocket (basic residues 41-43) was allowed for firm adhesion of S19 but did not trigger G-protein signalling. Only after the ligand had bound to the second site (aa131-133) did the receptor trigger a chemotaxis signal (Shibuya, 2001). The late appearance of S19 dimers, 24 hours after the heat treatment, suggests that this chemoattractive molecule may not be released as a result of apoptosis. It is probable that S19 had leaked out from disintegrating cells. This is contrast to EMAPII and LPC that are released very early on in the process of apoptotic cell death.

Endothelial monocyte-activating polypeptide II (EMAPII)

EMAPII was identified as a chemotactic molecule released from cells early on during apoptosis while the plasma membrane remained intact (Kao, 1994). EMAPII was isolated from (methylcholanthrene-A-induced) apoptotic fibrosarcoma cells and was found to be chemotactic to both monocytes and neutrophils. Like S19, EMAPII was also proinflammatory. It stimulated monocytes to release TNFα and also induced neutrophils to make myeloperoxidase (Kao, 1994). This was unexpected, since apoptotic-cell clearance is usually anti-inflammatory, although there have been a few reports of apoptotic cells generating a pro-inflammatory response (Albert, 1998). It is controversial as to whether mature EMAPII itself induces apoptosis or is cleaved from the enzyme complex by caspase-3 and caspase-7 during the process of apoptosis (Zhang, 2002).

Pro-EMAPII is homologous to the p43 subunit of the multi-enzyme complex, aminoacyl-tRNA synthetase (RS). Aminoacyl-tRNA synthetases are a family of enzymes that catalyse the attachment of specific amino acids to their cognate tRNAs and are vital for protein synthesis. The p43/proEMAPII subunit contains the RNA-binding portion of the complex. And similarly, Tyrosine-RS, Methionine-RS and

Phenylalanine-RS also contain EMAPII appendices. Tyr-RS was found in supernatants from apoptotic cells but Ala, Ile, Lys, Val-RS were not (Wakasugi, 2003). Proteolytic cleavage of p43/proEMAPII halted cellular protein synthesis and produced two fragments, both of which had chemokine activity. The C-terminal fragment of p43 (mature EMAPII) was chemotactic to monocytes and neutrophils. The other N-terminal-fragment, contained an ELR tripeptide sequence that was chemotactic for CXCR1-expressing neutrophils (Wakasugi, 2003). The N-terminal fragments of two other aminoacyl-tRNA transferases, Histidine-RS and Asparagine-RS, were also chemoattractant to activated (but not to resting) monocytes and immature DC's. Interestingly, Histidine-RS and Asparagine-RS are both autoantigens found in the autoimmune connective-tissue diseases: myositis and dermatomyositis (Howard, 2002). When aminoacyl-tRNA synthetases were released from damaged cells they might attract the very monocytes able to induced autoimmunity.

Lysophosphatidylcholine (LPC)

LPC is a phospholipid that was shown to be released from intact apoptotic cells, crucially, before disintegration of the plasma membrane. LPC was chemotactic to both monocyte cell lines and primary macrophages, although it attracted macrophages more efficiently (Lauber, 2003). Before its discovery as a chemotactic molecule, LPC was known to have a pro-inflammatory function. LPC is a major component of oxidised low-density lipoprotein and (like fractalkine) it may be an aetiological factor in arteriosclerosis. LPC caused inflammation associated with atherosclerotic plaques (Huynh, 2002) and it activated NFκB when it bound to its receptor, G2Λ (Kabarowski, 2001).

LPC appears to link together the caspase enzymes that regulate apoptosis, to the process of clearance of apoptotic cells. Lauber et al showed that LPC release depended on caspase 3. Although macrophages were able to engulf cells that died in the absence of caspase-3 (Turner, 2003), they did not move towards apoptotic MCF7 breast cancer-cells that lack casapse-3 (Lauber, 2003).Lauber et al showed that the apoptotic MCF7 cells could not attract monocytes, but chemotaxis was restored when Caspase-3 was transfected into MCF7 cells. The chemokine isolated from the supernatants of apoptotic cells was found to be the phospholipid,

lysophosphatidylcholine (LPC) already known to be a chemotactic molecule (Hoffman, 1982). Phospholipase A2 (PLA2) is the enzyme that catalyses the hydrolysis of membrane phosphatidylcholine into LPC and arachadonic acid. The PLA2 enzyme has many isoforms, but the cytosolic, calcium-independent form of PLA2 was the only one to be cleaved by caspases and so activated during apoptosis.

No doubt, LPC is an important link between apoptosis and macrophage chemotaxis to dying cells, but it is unlikely to be the only factor involved. Only a very small amount of LPC was found in the supernatants derived from apoptotic cells. In fact, much higher levels of LPC were required to attract macrophages than were found in the more potent supernatants.

Chemoattractive molecules and chemokines have important roles in directing the movement of macrophages. It has been seen that the persistence of apoptotic cells in the CD14-/- mouse did not cause inflammation. Indeed the high rates of apoptosis in Burkitt's lymphoma (BL) may benefit tumour growth. Whether the chemoattractive properties of apoptotic cells are able to draw in macrophages into tumours, like BL, will be discussed in the next section.

1.7 Macrophage infiltration of tumours

Macrophages are common cell components of tumours and their presence is associated with a poorer prognosis and a reduced 5 year-survival (Sikorsa, 2000). Resident macrophages in tumours and are generally referred to as Tumour-Associated-Macrophages (TAM) (Mantovani, 1992). Even though a "lymphoreticular infiltrate" was first noticed in tumours by Virchow in 1863 the role of TAM in tumourogenesis was not immediately apparent (Balkwill, 2001). Until recently, the tumour-associated macrophages found so commonly in Burkitt's lymphoma (BL) were described as "benign" in a standard pathology textbook (Cotran, 1989). Rather than being simply bystanders, TAM can influence the pathogenesis of tumours by multiple means. It has been suggested that "The inflammatory cells and cytokines found in tumours are more likely to contribute to tumour growth, progression and immunosuppression than they are to mount an effective host anti-tumour response" (Balkwill, 2001). This may be why it has proved so difficult to harness the immune system to destroy neoplastic cells.

TAMS produce many different factors that support all stages of tumour development from cell growth, longevity and survival, through proliferation, angiogenesis, invasion and finally to metastasis (reviewed in Mantovani, 1992; Mantovani, 2002). One of many examples of such tumour support is that TAM at low oxygen tensions found at the centre of a rapidly growing tumour produce vascular endothelial growth factor (VEGF) that promotes angiogenesis (Talks, 2000). The "starry sky" macrophages in Burkitt's lymphoma (BL) produce the B cell survival factor BAFF (B-cell activating factor of the TNF family) (Ogden, 2005). TAMs also aid tumour expansion and invasion by producing matrix-metalloproteinases that digest away elements of the extracellular matrix. Such TAM expression of MMP9 contributed to the growth of a skin cancer (Coussens, 2000). There is often a symbiotic relationship between the tumour cells and their associated-macrophage neighbours. For example, some tumour cells produce macrophage growth factors like M-CSF (macrophage-colony stimulating factor) that promote malignant behaviour as well as prolonging macrophage survival (Lin, 2001).

There is also evidence that some tumours secrete chemokines that specifically trigger macrophage infiltration. Tumour chemokines do more than just attract leukocytes: some CXC and CC chemokines can also stimulate angiogenesis and metastasis (Keane, 1999; Zlotnik, 2003). The chemokine CCL2 (MCP-1) is frequently found in solid tumours, but its effect depends upon its local concentration (Sica, 2005). Low levels of CCL2 recruited macrophages into a melanoma and promoted tumour growth. However, much higher levels of CCL2 caused a huge infiltration of macrophages that destroyed the tumour (Nesbit, 2001). Therefore, the chemokine balance within a tumour is critical in determining its pathogenicity (Mantovani, 1992)

"Classical" and "alternative" activation of macrophages

In addition to directly supporting tumour growth, TAMs help tumours to avoid the attention of the immune system. Most evidence shows that the tumour microenvironment is rich in the TH2 cytokines IL-4 and IL-6 and these skew TAM toward the "alternatively-activated" or M2 phenotype as opposed to "classically activated" or M1 type of macrophage (Mantovani, 2004; Gordon, 2003). A macrophage's response to infection by intracellular pathogenic mycobacteria, is an

example of the classical activation of M1 macrophages. TH1 helper T cells secrete interferon gamma (IFNγ) that classically arms macrophages to destroy infected cells. M1 macrophages also have the potential to kill tumour cells, however, most TAMs seem to express an M2-like phenotype (Mantovani, 2004).

The "alternatively activated" M2 macrophages have a different role from M1, consistent with humoral immunity against extracellular pathogens and also in angiogenesis, tissue modelling and repair. What is more, apoptotic cells appear to polarise macrophages towards an M2 phenotype leading them to secrete "type-2" cytokines like TGFβ and IL-10. In the presence of these cytokines, TAMs fail to instigate an inflammatory reaction to tumour antigens. In this way TAMs help tumour cells to avoid immune-surveillance and promote tolerance. For example, the IL-10–rich tumour environment of ovarian cancer induces TAM to produce CCL18. CCL18 is chemotactic to naive T cells, and in conjunction with M2 macrophages lacking a co-stimulation (signal 2) this results in anergy and a failure of the adaptive immune system to detect tumour antigens (Schutyser, 2002). The characteristic Reed-Sternberg cells are diagnostic in Hodgkin's lymphoma and secrete CCL22 and CCL17 that account for the T cell infiltration and yet doesn't lead to an immune response (van de Berg, 1999).

TAMs originate from circulating blood monocytes and there is not much evidence to suggest that *in situ* proliferation sustains the population. In the face of recruitment of fresh potentially inflammatory monocytes the tumour microenvironment is especially important in driving the recruited monocytes toward an M2 phenotype (Mantovani, 1992). One tumour where the tissue microenvironment is rich in IL-10 is Burkitt's lymphoma (BL) (Levens, 2000).

1.8 Macrophage infiltration of Burkitt's lymphoma

Burkitt's lymphoma (BL) is a high grade, B-cell non-Hodgkin's lymphoma. In its endemic form, BL often affects the jaw of children living in the malaria-affected countries of West Africa and Papua New Guinea. Tumours from this geographical location are often associated with Epstein Barr Virus (EBV) infection (Burkitt, 1958; Berard, 1969). The sporadic form of BL if found more globally, outside of the endemic areas and is not usually associated with EBV. BL is a

neoplasm originating from germinal centres B-cells (Gregory, 1988; Chapman, 1998). BL contains frequent apoptotic cells and infiltrating macrophages, which endow the tumour with its classical 'starry sky' histological appearance (Wright, 1970). The numerous pyknotic nuclei that are found in the tumour are testament to the high rate of apoptosis that accompanies cell proliferation. In figure 4.1a, tumour-associated macrophages are found actively engulfing the numerous apoptotic cells they encounter and consequently, their phagosomes are engorged with apoptotic bodies.

c-Myc

Apoptotic cells are a consistent feature of BL. The high rate of tumour growth may limit the availability of nutrients and growth factors causing high rates of spontaneous apoptosis (Harrington, 1994). In addition, the presence of apoptotic cells is due to complex interactions between c Myc and EBV (Fujita, 2004). The *c-myc* gene product Myc, is a gene-regulatory protein that stimulates cell division. In BL, chromosomal translocations, most commonly, t8:14 and t2:8, result in the juxtaposition of *c-myc* next to powerful genes controlling immunoglobulin expression. Thus, Myc regulation becomes defective in B-lymphocytes, the cells that normally produce antibody. As well as stimulating cell division, Myc also drives the constitutively high rates of apoptosis (Juin, 2002). c-Myc causes mitochondrial triggering of apoptosis mediated through the pro-apoptotic cytochrome C and antagonises the anti-apoptotic members of the Bcl-2 family (Moreau, 2003). Anti-sense oligonucleotides that blocked expression of Myc could also enhance the survival of BL group-1 cells in a similar way to interferon-alpha (Milner, 1993).

p53

One of the most important checks against tumour proliferation is provided by p53, a gene regulatory protein that can be mutated in approximately 60% of BL derived cell-lines (Banthia, 2003; Wiman, 1991). p53 is mutated in the BL-cell line "Mutu" used in this work. "Mutu" BL-cells are heterozygous for p53 and expresses one mutant copy and one wild-type copy of the p53 gene (Milner, 1997). DNA damage, say from UV-light, leads to the activation protein kinases that stabilise the p53 gene product and prevent its breakdown. High concentrations of p53 can

stimulate many of the genes important in cell–cycle arrest and apoptosis. The re-addition of a wild type p53 back into one (of the many BL) lines that had mutated p53, resulted in apoptosis (Ramqvist, 1993; Wang, 1993). Interestingly, the chemokine fractalkine has also shown to be a direct target of p53 and potentially could be up-regulated during apoptosis (Shiraishi, 2000). Production of fractalkine during apoptosis may attract CX3CR1-expressing cells like macrophages, important for clearance.

Epstein Barr Virus

Net growth of BL-tumours is a balance between rates of proliferation and apoptosis. EBV infection is common, and infects approximately 90% of the world's population. It is one of the causative factors in Burkitt's lymphoma (van den Bosch, 2004). EBV is important for BL-survival because it can prevent apoptosis triggered by p53 and myc by the up-regulation of bcl-2 (Okan et al, 1995). This work and work carried out previously in the group showed that the transfection of Bcl-2 into BL-cell lines could reduce both spontaneous and UVB-triggered apoptosis (Hong Wang PhD thesis).

When EBV-positive BL cell-lines are cultured up from original tumour biopsy cells, they can dramatically change their phenotype (Rooney, 1986). They acquire lymphoblastic characteristics that are similar to EBV immortalised cells that have escaped T-cell control. Freshly established BL lines have been described as having a "group I" phenotype close to their *in vivo* tumour origin and similar to germinal centre B cells. Interestingly, BL-cell lines that have not been infected with EBV, retain this more stable, group I phenotype. BL-cells in group I do not express the EBV antigens EBNA-2 (EBV nuclear antigen-2) and LMP (latent membrane proteins). As BL-cells progress towards a lymphoblastoid like phenotype begin to express LMP and EBNA-2. Cells displaying these EBV antigens tend to clump together in culture and become resistant to apoptosis are termed "group III" cells. The BL-cells with an intermediate phenotype are termed group II (Rooney, 1986; Rowe, 1987). EBV infected cells must gain access to the B memory pool in order to survive. Normal B cells, which have not encountered cognate antigen will not become memory cells and will die by apoptosis. The expression of EBV latent

proteins protects B cells from apoptosis and smoothes their path into the memory pool (Gregory, 1991; Milner, 1997; Okan, 1995).

Apoptosis in Burkitt's lymphoma

Interactions between EBV infection, p53 and Myc predispose to apoptosis in BL (Harrington, 1994). The accumulation of apoptotic cells in BL and also in the CD14-/- mouse is striking. This thesis will investigate whether apoptotic cells in BL are more than just the natural waste products of a successful tumour and if apoptotic cells can themselves confer any survival advantage. The apoptotic cells may support the tumour indirectly through interactions with tumour-associated macrophages. The "starry sky" macrophages provide survival factors such as BAFF/BLyS (B cell activating factor of the TNF family/B lymphocyte stimulator) that ensure that tumour cell growth exceeds apoptosis (Ogden, 2005). EBV-positive tumours produce viral homologs of IL-10 and in turn, this cytokine increases the efficiency of macrophage engulfment of apoptotic cells (Alcami, 2003). TAM in both BL and a fibrosarcoma down-regulated the production of IL-12 and NF-κB in response to IL-10 (Ogden, 2005; Sica, 1997). Macrophage phagocytosis of apoptotic cells is often accompanied by the release of anti-inflammatory mediators like TGF-β, PGE2 and PAF that could also suppress anti-tumour immunity (Voll, 1997; Fadok, 2001; Fadok, 1998; Kurosaka, 2003).

Despite their large numbers, apoptotic cells do not appear to result in inflammation in BL. This thesis will examine whether apoptotic cells are instrumental in attracting macrophages into BL-tumours.

Summary

The study of Burkitt's lymphoma cells has provided us with much information about apoptosis (Milner, 1993; Gregory, 1987; Gregory, 1994). The close association of macrophages and apoptotic cells within this tumour make it an ideal model for the study of the interactions between phagocytes and dying cells. In the presence of IL-10, the "starry sky" TAMs are transformed into professional phagocytes "par-excellence". This enhanced ability to engulf dying cells is a process dependent on the tethering molecule CD14 (Devitt, 2004). The observation that many of the apoptotic cells in the lymphoid organs of the CD14-/- mouse were found

to be "free", that is, unattached to a macrophage is very interesting. It suggests that CD14 may have a role in the "come-get-me" stage of engulfment, i.e. in macrophage chemotaxis to apoptotic cells.

This work explores the role of apoptotic cells in the recruitment of tumour-associated macrophages into Burkitt's lymphoma. This may have some relevance to macrophage chemotaxis within the physiological setting of the germinal centre and could shed light on generic mechanisms of macrophage recruitment to tumours.

1.9 Aims

To determine if CD14 is involved in the attraction to, and uptake of, apoptotic BL-cells. In order to do this:

(A) This work will enumerate the apoptotic cells in the thymus of the CD14-/- mouse compared to the wild type on both the BALB/c and C57BL/6 strain backgrounds.

(B) Measure interaction of CD14-/- macrophages with apoptotic BL-cells *in vivo*, compared to wild type macrophages.

(C) Characterise $CD14^{low}$ human macrophages' interaction with apoptotic BL-cells compared to $CD14^{high}$ macrophages.

(D) Measure macrophage migration to apoptotic BL-cells in a transmigration assay

(E) To assess the importance of CD14 and CD36 in this process using bone marrow-derived macrophages from the CD14 and CD36 knockout mice compared to wild type.

Finally the identity of the macrophage chemoattractant molecules released by apoptotic BL-cells will be sought.

CHAPTER 2

Materials and methods

2.1 Preparation of human primary cells

The Lothian regional ethics committee approved the use of buffy coats and donated blood in this work. All procedures involving humans were carried out according to the strict health and safety-guidelines of the University of Edinburgh. Human peripheral blood was obtained from normal volunteers and buffy coats were obtained from the Scottish National Blood Transfusion Service.

PBMC from buffy coats

The whole volume of a single donor buffy coat (approximately 80-120ml) was made up to 200ml by dilution with PBS. 25ml of diluted blood was carefully laid over 15ml of Histopaque 1077 (Sigma) in a 50ml sterile tube (Falcon) and centrifuged for 30 min at 2220 RPM/600g(brake off). The second layer, containing the white blood cells (wbc) was carefully pipetted off and washed in 200ml PBS and re-spun at 1200 RPM/400g for 10 min. If the supernatant appeared cloudy, the cells were washed again to remove contaminating platelets. PBMC were purified by adherence to plastic. 5x 10^6 PBMC in $IMDM_{supp}$ for 1 hour at 37C (without serum) and were plated onto 6 well plates (Costar, UK). Afterwards, the non-adherent lymphocytes were removed and the media replaced with $IMDM_{supp}$ containing 10% human AB serum (Sigma).

PBMC from peripheral blood

40ml of whole blood was placed into a 5oml centrifuge tube containing 4.4ml of 3.8% sodium citrate and spun at 1200 RPM/400g for 20 minutes with the break off. After spinning the upper layer that contained plasma and platelets was removed and spun art high speed (3000 RPM/800g) to separate the platelets from the plasma. Plasma proteins were allowed to clot around a sterile paper clip in the presence of a final concentration of 40mM $CaCL_2$ at 37C.The resulting platelet poor plasma (PPP) was saved for later use. The erythrocytes remaining in the blood were allowed to sediment for 30 minutes in the presence of approximately 5ml (10% of blood volume) of 6%dextran in 0.9% (normal) saline. The wbc in the upper layer were removed using a pipette and centrifuged for 6 minutes at 960 RPM/300g. The resulting pellet of wbc, that was contaminated with some erythrocytes, was

resuspended in the PPP already prepared. 2ml of PPP was added for every 80ml of wbc. A Percoll gradient (Amersham Pharmacia Biotech, UK) was prepared as follows: A sterile, 90% Percoll solution was made up in normal saline and kept at 4C. A 42% Percoll solution was made up by adding 0.84mlof 90% Percoll to 1.16ul of PPP and was vortexed. A 51% Percoll solution was made up by adding 1.02mlof 90% Percoll to 0.98 of PPP and was vortexed. The resuspended wbc were placed in a 15ml conical tube and first the 41%, followed by the 51% Percoll solutions were under-layed beneath the wbc. The gradient was spun for 10 minutes at 1000 RPM/300g (brake off). Peripheral blood mononuclear cells (PBMC) were removed from the upper layer and neutrophils from the middle layer, and any remaining erythrocytes were discarded. Neutrophils were washed in PBC and used immediately in chemotaxis assays. PMBC were washed in PBS and further purified by either magnetic cell sorting (Miltenyi Biotech, Germany) or by adherence to plastic. 5x 10^6 PBMC in $IMDM_{supp}$ without serum were plated onto 6 well plates (Costar, UK) at 37C for 1hr and the non-adherent lymphocytes were removed and the media replaced with $IMDM_{supp}$ and 10% heat-inactivated autologous serum or alternatively, human AB serum (Sigma). The sorting of CD14-positive and negative monocyte fractions from PBMC was done exactly according to the manufacture's instructions (Miltenyi Biotech).

Preparation of neutrophils from peripheral blood

Peripheral blood mononuclear cells (PBMC) and neutrophils were isolated from the blood of normal donors by separation over a Percoll (Amersham Pharmacia Biotech, UK) gradient as previously described. Neutrophils were washed in PBS and used immediately. Mononuclear cells were plated at 5x 10^6 cells/ml in 6 well tissue culture plates (Costar). Adherent monocytes were either used immediately or matured into macrophages by culture for a further 8 days in $DMEM_{supp.}$ and heat inactivated 10% autologous serum or human AB serum (Sigma).

Separation of $CD14^{low}$ and $CD14^{high}$ human monocytes.

A combination of magnetic cell sorting (MACS) and adherence was used to isolate $CD14^{high}$ and $CD14^{low}$ monocytes from PBMC. PBMC were isolated from the blood of normal donors by separation over a Percoll gradient as previously described.

Anti-CD14MACS beads (Miltenyi Biotec, Germany) were used to select CD14 positive monocytes from PBMC (Milteny Biotech) exactly according to manufacturer's instructions. CD14 positive and negative cells were automatically separated over magnetic columns (Automax, Miltenyi Biotec, Germany). The CD14-positive fraction was washed and resuspended to a concentration of 10^6 monocytes/ml in $IDDM_{supp}$ without serum for 1 hour. The CD14-negative fraction (that had not bound the anti-CD14 labelled magnetic bead) was washed and resuspended to a concentration of $5x10^6$ cells/ml in DMEM without serum for 1 hr. Cells were plated into 6-well plates for FACS analysis, 48-well plates for phagocytosis assays (Costar, UK) and into 0.5 ml chamber glass slides for immunohistochemistry. After a 1 hour incubation, media and non-adherent cells were removed and the media was replaced with $IDDM_{supp}$ with 10% serum. Cells were harvested at Days 0, 1, 3, 5 and 7 for FACS and immunohistochemical analysis. Macrophage interaction with apoptotic cells was assayed using 10 day-old macrophages in 48-well plates.

Burkitt's lymphoma

The Burkitt's lymphoma tumour biopsy specimen was a gift from Katrina Wood, Department of Pathology, Royal Victoria Infirmary, Newcastle upon Tyne and The University Newcastle upon Tyne.

2.2 Maintenance of human cell lines

Burkitt's lymphoma cell lines, Mutu and BL2

The BL-cells, Mutu and BL2 were cultured in suspension in RPMI 1640 supplemented with 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin ($RPMI_{supp.}$) and 10% serum supreme (Biowhittaker, MA) (Gregory, 1990).

Mono Mac 6

MM6 cells were cultured in DMEM supplemented with 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin ($RPMI_{supp.}$) and 10% serum supreme (Biowhittaker, MA) (Ziegler-Heitbrock, 1988).

Transfection of Mutu and BL2 with *bcl-2*

Mutu were transfected with the plasmid pEF*bcl-2*MC1neopA by Hong Wang using a method described in his thesis "Regulation of death and survival in Burkitt's lymphoma cells" Nottingham University 1996 (Visvader, 1992). BL2 were also transfected with the plasmid pEF*bcl-2*MC1neopA by Carol Anne Ogden. BL-cells that had been transfected with *bcl*-2 (Mutu $_{bcl\text{-}2}$ BL2 $_{bcl\text{-}2}$) were cultured with RPMI $_{supp}$ containing 2.5mg/ml neomycin (Gibco Invitrogen, UK). Cells were passaged every 2-3 days and were maintained at an average concentration of 10^6 cells/ml.

Cryopreservation of cell lines

Cell lines required for future use were washed and resuspended in 1ml FCS and then mixed with 1ml of 90% FCS/10% dimethyl sulfoxide (DMSO). Cells were aliquoted into cryotubes and frozen slowly first in the –70C freezer for 48hr and then transferred into liquid nitrogen for longer-term storage. To thaw cells, cryo tubes were wrapped in aluminium foil and left at room temperature. Once defrosted, cells were washed several times in medium to remove residual DMSO.

2.3 Preparation of mouse primary cells

All animal work was performed under licence from the UK Government Home Office and with the approval of the review boards of the Universities of Nottingham and Edinburgh.

Mice

Balb/c wild type and the C57BL/6 wild type mice were purchased from B&K Universal (Hull,UK). Balb/c CD14-/- were produced as described by (Haziot, 1996) and were provided by Sanna Goyert, Northshore New York. The Balb/c mice were held in conventional housing at the University of Edinburgh.

C57BL/6 CD14-/- mice were obtained from Jackson laboratories (Jackson, Maine USA) and were certified as being specific pathogen free and were housed in individual ventilator cages. C57BL/6 CD36-/- animals were a kind gift from Professor John Savill, University of Edinburgh, and were held in conventional housing at the University of Edinburgh.

Mouse bone-marrow-derived macrophages (BMDM)

Mouse macrophages were cultured from bone marrow using previously described methods (Ren, 2001; Savill, 1992; Johnson, 1983). Entire femurs were removed from mice using a sterile scalpel, by disarticulation at the hip and osteotomy through the proximal tibia and fibula. Bones were placed in 100% alcohol for 5 minutes. Muscle was cleaned away from bone and the shaft of the femur was cut at each end. Marrow flushed out of the femur using a small gauge (orange) needle connected to 5ml syringe containing with DMEM supplemented with 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, ($DMEM_{supp}$)(Gibco, Paisley, UK). The cells were dispersed by drawing them up and down through a medium gauge (green) needle. Bone marrow cells were washed in 10ml $DMEM_{supp}$ and centrifuged at 1200 RPM/400g for 5 minutes. Cells were stained with trypan blue and counted using a glass haemocytometer; they were resuspended at a concentration of 10^{6} cells/ml in $DMEM_{supp}$ containing 10% heat inactivated FBS and 10% conditioned supernatant from L929 cells as a source of M-CSF (Labtech International, UK) (Hume, 1983).

Bone marrow cells were plated out into sterile tissue culture plastic plates (Costar UK). Macrophages to be used for chemotaxis assays were seeded in 6-well plates and those to be used for phagocytosis assays were seeded into 48-well plates. Cells were maintained in an incubator kept at 37Cand 5% CO_2. After 24 hours in culture, supernatants containing non-adherent cells were removed and adherent macrophages were washed in PBS and then fresh media was added. Bone marrow-derived macrophages (BMDM) were cultured for up to two weeks and fresh media was supplied after every three days in culture.

Mouse thymus for transmission electron microscopy (TEM)

Wild type and mice and CD14-knockout mice were used between 6- 8 weeks of age. One wild type mouse and one CD14-/- mouse were injected with 200μg of dexamethasone ip (Organon,UK) 24 hours prior to sacrifice, and a second pair of mice were injected with PBS vehicle control. Each thymus was briefly examined before processing for TEM. 4 x $2mm^3$ pieces of thymus were cut from the remaining lobe. Both the cortex and medulla regions of the thymus were sampled. Tissue was

immediately fixed in freshly glutaraldhyde. Mr Stuart McKenzie from the Department of Pathology of the University of Edinburgh and the Lothian University Hospitals Trust kindly prepared the specimens for TEM. Briefly, specimens were dehydrated, and embedded in resin and sections were cut and mounted on copper grid. Specimens were viewed using the electron microscope at the Western General Hospital, Edinburgh and photographs were taken of representative material. Black and white photographs were scanned and saved as JPEG files.

Mouse thymocytes for FACS

After sacrifice, the thymus was removed. Thymus tissue was squashed between two glass slides. The liberated cells were suspended in PBS in a 15ml tissue culture plastic tube. Dense thymus tissue fell to the bottom of the tube and the single cell-suspension above was aspirated, spun down at 1200 RPM/400g and resuspended in Annexin V binding buffer ready for staining with Annexin V FITC (AxV) and propidium iodide (PI). Cells were analysed by FACS, 10 000 events were counted and the total percentage of AxV- PI- (viable cells) versus the AxV+ PI+ (apoptotic cells) was compared for each mouse.

2.4 Immunohistochemistry

Haematoxylin and eosin immunostaining of cytospins

Cells were washed and resuspended in RPMI or PBS with 10% serum supreme (Biowhittaker, MA) at a concentration of 2.5 x10^5 cells/ml. 200ul of the cell suspension was added to each sample chamber. Poly-L-lysine coated or BDH superfrost-plus glass slides were used. The cells were spun on a Shandon Cytospin3 (Shandon Inc. USA) at 75g for 3 min. Cells were left to air-dry and were fixed in methanol for 2 min, eosin stained in Diff-Quik1 for 2 min followed by haematoxylin staining in Diff-Quik 2 (Dade UK) for one minute and then immediately rinsed in water. After drying for 10min cytospins were wetted in xylene and mounted in pertex mountant (Cellpath, UK).

Deoxynucleotide transferase-mediated dUTP nick end labelling (TUNEL) staining of fixed tissue

A TdT-FragEL ™ DNA fragmentation kit (Oncogene research products) was used for TUNEL staining which labels the brakes in the DNA found in apoptotic cells. The manufacturers instructions for the kit were followed exactly, except that one drop of reagent A was added per ml of reagent B of DAB liquid (Dako) and was used in place of the kit's DAB 0.7mg tablet.

10mM Tris pH 8 was made up as follows. 1.211g of Tris was dissolved into one litre of dH_2O, and HCL was then added to reduce the pH to 8.0. The pH was measured using a pH meter. 1mM $MgSO_4$ was made up as follows. 247mg $MgSO_4$ was dissolved into one litre of 1x TBS. The kit's proteinase K was diluted 1:100 from concentrate. 1ug/ul DNAse-1 was made up as follows: 10mg/ml of DNAse-1 (Sigma) was diluted 1:10 in 1mM $MgSO_4$ in 1xTBS. Hydrogen peroxide was made up as follows. 1ml of 30% H_2O_2 was diluted 1:10 in 100% methanol to a final concentration of 3% H_2O_2.

After mounting a glass cover slip over the samples, the number of positive cells that stained brown in the cortex and the medulla were counted. Three representative sections each 5μm thick were taken 50μm apart through the whole thickness of the thymus from each mouse. For each section, four randomly selected, non-overlapping high power fields were selected. Each field was 100μm x 100μm. A HOME ™ computer with Axio-home software (Zeiss) was used to keep a tally of positive cells and to ensure that positive cells were counted only once. The mean number of cells in each high-power field was counted and all data was graphed using Graph Pad Prism software version 3.02.

CD68 immunohistochemisty

The human Burkitt's lymphoma tumour biopsy specimen was a gift from Katrina Wood, Department of Pathology, Royal Victoria Infirmary, Newcastle upon Tyne and The University Newcastle upon Tyne. The formalin-fixed, paraffin-embedded Burkitt's lymphoma tumours were cut into 4 μm-thick sections using a microtome and mounted onto Vectabond-coated glass slides (Vecta, CA

Paraffin-embedded slides were dewaxed in xylene, for 10 minutes at room temperature, then rehydrated to water by 2 minute sequential washes in absolute alcohol, 74% alcohol, then 64% and finally deionised water. If antigen retrieval was required, slides were placed in a plastic dish (with a lid) containing Vector antigen unmasking solution (Vector, USA) that had been diluted 1:100 in deionised water. Slides were microwaved for 3x 5 minutes at 1000 watts. The slide rack was turned to disperse air bubbles after each 5-minute cycle. Slides were then cooled in running tap water for 20 minutes. Non-specific staining was blocked using 0.15% H_2O_2 diluted in tap water for 15 minutes. Slides were washed twice for 5 minutes in PBS and loaded onto a Shandon Sequenza staining rack that prevented tissue drying (Shandon,USA). The Sequenza reservoir had a capacity of 125ul and all antibodies and solutions were made up to a volume of 125ml. Sections were blocked with Vector Avidin D blocking solution for 15 minutes, washes twice in PBS for 5 minutes and then further blocked with Vector Biotin solution for 15minutes followed by 2 5-minute PBS washes. A 1:50 dilution of a mouse anti-human CD68 monoclonal antibody clone PG-M1 was made into a 5% goat serum (Harlan Sera-Lab) in PBS containing 4 drops of biotin block per/ml. Slides were incubated for 1 hour at room temperature, washed in PBS and then incubated with a secondary antibody. A biotinylated goat anti-mouse Ig monoclonal antibody (Dako) diluted 1:300 in PBS containing 5% normal goat serum and biotin block was incubated for 30 min.

After staining with specific antibodies, slides were washed with PBS followed by a 30-minute treatment with R.T.U Vectastain Elite ABC reagent, that contains a preparation of streptavidin-horse radish peroxidase (HRP). After three 5-minute washes in PBS, antibody labelling was visualised using a diaminobenzidinetetrahydrochloride (DAB) substrate solution for 5 minutes. DAB was freshly prepared immediately prior to use by adding one drop of liquid (20µl) DAB to 1ml of substrate chromogen solution (Dako Liquid DAB kit, Dako, Denmark). Slides were removed from the Sequenza rack and washed in PBS. Tissues were counterstained blue in haematoxylin (Shandon) for 30 seconds and then dipped in Scott's tap water for 30 seconds. Slides were then dehydrated in ascending

concentrations of alcohol (same% as before). Slides were cleared in xylene and mounted with a Pertex mountant and protected with glass coverslips.

Confocal fluorescent microscopy of CX3CL1-labelled cells

A confocal microscope can focus on ultra-thin sections of a cell. Only when the laser is focused to a high resolution is the light sufficient to excite fluorescence. Thus the sections viewed are thinner than the cell width. A confocal microscope has an advantage over light microscopy because it can differentiate between surface and intracellular molecules.

Mutu BL-cells were incubated with a mouse anti-fractalkine antibody clone 81513 (R&D systems) and bound antibodies were revealed by incubation with a secondary, goat anti-mouse Igalexa-fluor green 488-labelled antibody. Cells were fixed in 1% paraformaldehyde at 4C overnight and suspended in a 50:50 PBS/glycerol mix containing 1% vector shield (Vector) and finally, mounted with Movoil for confocal microscopy. Cells were viewed using a TCS-NT confocal laser scanning microscope (Leica Microsystems) coupled with Ti:Sapphire lasers. Images were obtained with the kind assistance of Linda Wilson at the University of Edinburgh.

2.5 Fluorescence-activated cell sorting

Fluorescence-activated cell sorter (FACS) analysis of cells is used to characterise single cells within a suspension. When cells are labelled with fluorescent antibodies, the machine is able to measure the size, granularity and fluorescence of an individual cell as it passes in a stream across photodetectors. FACS analysis of cells was carried out using either a Coulter EPICS XL (Coulter) or a Becton Dickinson FACS Calibur (BD Immunocytometry systems).

For fluorescent antibody labelling, approximately $2x10^5$ cells were added to 96 well round-bottom tissue culture plates (Costar) for each test, and washed in 200µl of FACS buffer, and spun down at 1200 RPM for 5 minutes at 4C. Cells were then resuspended in 100µl of cold FACS buffer and placed on ice. For single step, direct labelling of cells, FITC, RPE or RPE-Cy5 conjugated antibodies or isotype control antibodies were added to cells and incubated on ice in the dark for 20

minutes. The concentration of antibody added varied depending on the manufacturers recommendations and the optimum antibody concentration was titrated for each cell type used. Two-step, indirect labelling of cells was done when the primary antibody was not conjugated to a fluorochrome. After the first incubation, cells were washed and incubated with a secondary antibody conjugated with FITC, RPE or RPE-Cy5. Secondary antibodies were either full length or Fab fragments of antibodies raised against the primary immunoglobulin.

Cells were washed twice in cold FACS buffer and then resuspended in 400µl cold FACS buffer and used immediately. Propidium iodide (PI) and (ToPRO3) are two intracellular dyes that are taken up by dead and fixed cells. 1µl of PI or ToPRO3 was added immediately to cells before running on the FACS machine in order to discriminate dead from viable cells. If cells were not be analysed immediately, they were fixed in a 50:50 mixture of 200µl FACS buffer and 200µl of FACS fix buffer to a final concentration of 1% formalin. Cells were then stored at 4C in the dark until further use. Cells were analysed by a Coulter EPICS XL FACS machine and data files were analysed using Flow-Jo software.

2.6 Molecular Biology

Preparation of total RNA

An RNeasy Mini Kit (Quiagen) was used to extract total RNA from Mutu BL-cells, macrophages and monocytes according to the manufacturer's instructions. A maximum of 10^7 cells were suspended in an appropriate volume of the supplied RLT lysis buffer. Cells were then homogenised by passing through QIAshredder coloums (Quiagen). One volume of 70% ethanol was added to the homogenised lysate prior to being added to a Rneasy spin column. The lysate was centrifuged at 13,000 RPM/16060g for 15 sec and the column washed with the supplied RW1 buffer. Contaminating DNA was removed using RNAse-free DNAse (Quiagen). The column was washed twice in supplied RPE buffer. Total RNA was eluted in 20-30µl of RNAse/DNAase-free water (Promega Corporation).

The total amount of RNA was measured using a spectrophotometer, Ultrospec 200 (Pharmacia Biotech). The absorbency of known dilutions of extracted

RNA were measured at 260 and 280nm. These readings were converted to concentrations using the following formula (Sambrook, 1989):

1 A_{260} unit of RNA = 40μg/ml

To determine the purity of the RNA solutions, the ration of absorbance at 260nm and 280nm ($OD_{260.280}$) was measured. Ideally this value should be 2.0 for RNA. Running 2ul of the diluted RNA sample on a 1% agarose gel for 25 minutes checked the quality of the RNA sample. The resulting gel showed good 28s and 18s bands. Additionally, any contamination of the RNA sample with DNA was checked by running a PCR in the absence of reverse transcriptase (RT). These controls were necessary as many chemokine receptors are encoded by single exons, meaning that any contamination with genomic DNA may lead to a false positive result.

RT-PCR

CDNA was prepared using an access RT-PCR system kit A1250 (Promega). The reverse transcriptase enzyme used was RT superscript™ (InVitrogen) because it had no endonuclease activity and gave higher cDNA yields. 0.5μl of RNAse inhibitors were added to 5μg of RNA to a final volume of 7μl in RNAse/DNAase-free water (Promega Corporation). This was placed in a sterile, RNAse/DNAase-free, thin-walled tube and denatured for 5 minutes at 94C. The denatured sample was placed on ice and the following added:

4μl	25mM $MgCl_2$
4μl	5x RT buffer
2μl	10mM dNTP
0.5μl	RNase inhibitors
0.25μl	random hexamers
0.25μl	oligo dTs
2μl	RT enzyme

to a final volume of 20μl. Tubes were placed in a thermocycler (Perkin Elmer) at 42C for 1 hour followed by 95C for 5min and 4C for eternity. After diluting the cDNA sample to 100μl volume by adding 80μl of RNAse/DNAse-free water, samples were stored at –20C until further use.

PCR

Fractalkine, fractalkine receptor and β-Actin primers were a kind gift from Dr Robert Nibbs, University of Glasgow. All PCR was performed in the Beatson laboratory in Glasgow.

Primers

All primers were designed by Dr Robert Nibbs and manufactured by TAGN Ltd and MWG-Biotech, Glasgow.

CX3CL1 forward: 5'-CCA CGG TGT GAC GAA ATG-3'
CX3CL1 reverse: 5'-CCA TTT CGA GTT AGG GCA-3'

CX3CR1 forward: 5'-GAA ATC TGG CCC GTG CTC-3'
CX3CR1 reverse: 5'-CGG TTG CAT TTA GCC ATT G-3'

β-Actin forward: 5'-TCC ATC ATG AAG TGT GAC GT-3'
β-Actin reverse: 5'-TAC TCC TGC TTG CTG ATC CAC -3'

Primers were diluted in RNAse/DNAse-free water to a final concentration of 10μM. Pre-aliquoted Reddy mix Master Mix 0.2ml tubes (AM337-415 Abgene) containing loading dye, were used for PCR. 1.25μl of forward primer, 1.25μl of the reverse primer and 2.5μl of cDNA were added to the master mix. Water only and genomic DNA were added in place of cDNA as controls. Tubes were vortexed gently and placed on ice. cDNA was amplified in a thermocycler (Perkin Elmer) under the following conditions:

Hot start	94C for 5 minutes	
And 40 cycles of:	94C	20 seconds
	58C	1 minute
	72C	2 minutes
	72C	10 minutes
And afterwards	4C for eternity	

Products from this reaction were kept at 4C for approximately 48 hours until further use. Amplified products were separated out on a 1.5% agarose (Flogen) gel

containing 10µl ethidium bromide. 20µl of amplified product was run per well and fragment size was read using a coloured marker (Bioline Hyperladder-1). After approximately 45 minutes, gels were removed from the tank and viewed under UV light and photographed.

2.7 Western Blotting

Preparation of cell lysates

All steps were carried out at 4C using ice-cold solutions. Live and apoptotic Mutu BL-cells were washed twice in PBS and spun down. The 0.5ml of extraction buffer was added per 0.5x 10^6cells in the pellet. %). 50x Extraction Buffer was made up as follows: 20mM Tris-HCl (0.242g for 100ml) 2mM EGTA (0.076g for 100ml) and 2mM EDTA. The pH was adjusted to 7.5 with HCl This mixture was aliquoted and stored at –20C. 10ul of Triton x-100 was added to 10ml of a 1:50 dilution of the extraction buffer to a final concentration of 0.1% v/v. One tablet of protease inhibitors was added to 10ml of the buffer. The lysed cells were centrifuged at 3000 RPM for 10 minutes at 4C. 2µl of protein extract was added to 798µl of dH_2O. The total protein concentration in the samples was compared to a BSA standard concentration curve measured using a Bradford protein assay. A BSA standard curve of 2,4,8,12,and 20 µg/µl was prepared by adding 1,2,4,6 and 10µl of a standard stock solution (2µg/ul BSA) to 799,798,796,794,and 790 µl (respectively) of dH_2O. A blank reference sample was prepared by adding 2µl of buffer to 798µl of dH_2O. Whilst stirring on a vortex, 200µl Bradford reagent (Biorad) was added to each tube and the colour reaction proceeded for 10 minutes. 200µl of each sample was next placed in a 96 well flat-bottomed plate and read at 540nm in a plate reader (Anthos Labtec Instruments). A standard curve was plotted and the protein concentration of the samples read off. All protein samples were diluted to 5 µg/ µl in sample buffer.

8ml of 4x sample buffer was prepared by adding 1ml of 0.5M tris-HCL pH6.8 to 3.8ml of dH_2O, 0.8ml of glycerol, 1.6ml of 10% (w/v) SDS, 0.4ml beta-mercaptoethanol and 0.4ml of 1% (w/v) bromophenol blue. Samples were denatured by boiling in a x1 concentration of sample buffer for 5 minutes.

SDS-polyacrylamide gel electrophoresis

Gels were prepared as follows: A 12% separating gel was made up using 3.35ml dH_2O plus 2.5ml Tris-HCL pH 8.8, 100µl of 10% SDS, 4ml of 30%Acrylamide/bisAcrylamide, 150µl of 10% Ammonium Persulphate and 5µl TEMED. A 4% stacking gel was prepared as follows: 6.1ml dH_2O plus 2.5ml Tris-HCL pH 6.8, 100µl of 10% SDS, 1.3ml of 30%Acrylamide/bisAcrylamide, 150µl of 10% APS and 10µl TEMED. The separating gel was poured between two sheets of the Bio-rad mini gel apparatus. Once firm, the stacking gel was poured over a comb ensuring that no air bubbles became trapped.

A running buffer was prepared as follows: To make a 10x PAGE buffer 144g of glycine and 30g of Tris Base were added to 1 litre of dH_2O. The running buffer was prepared by taking 80ml of the PAGE buffer and 8ml of a 10% SDS solution and adding 912mlof dH_2O. The reservoir of the Bio-rad apparatus was filled with running buffer and the samples were loaded alongside a broad range molecular weight marker ranging from 10 to 250. The 8kDA chemokine domain fragment of fractalkine was added to one lane as a positive control. Two samples were run on a separate gel and tested for β-actin as a positive control of sample loading and protein content. Gels were run at 50mAmps for approximately 45 minutes.

Transfer of proteins from SDS-polyacrylamide gels to a solid support

Once the proteins had separated, gels were immobilised onto nitrocellulose paper (Amersham Biosciences) soaked in blotting buffer and sandwiched between layers of filter paper (Whatman) and sponge in the Bio-rad mini-Protean apparatus. The blotting buffer was prepared by adding 100ml of the x10 PAGE buffer to 200ml of methanol and adding 700ml dH_2O. Proteins were transferred from the gel to the membrane at 400mAmp for 30 minutes. Once transferred, the nitrocellulose papers were placed in a petri dish and stained.

Antibody labelling of protein bands

Proteins were blocked for 2 hours at room temperature using a 5% solution of non-fat skimmed milk powder (Tesco) made up in TBS. Membranes were labelled with a mouse anti-fractalkine monoclonal antibody clone 815 13 at a concentration 2µg/ml 1:250 (R&D Systems) or with a 1:500 mouse anti β-actin antibody (Sigma)

and incubated for either 2 hours at room temperature or overnight at 4C. The secondary antibody, biotinylated goat anti-mouse IgG antibody was diluted in TBS containing 5% milk to a ratio of 1:10 000. The secondary antibody was incubated for either, 1 hour at room temperature or overnight at 4C. Membranes were washed in TBS and bands revealed using enhanced chemiluminescence (ECL) (Upstate Biotech). The ECL solution was prepared by adding 1ml of solution A to 1ml of solution B per membrane for 1 minute. Membranes were wiped, wrapped in cling film and exposed to film. Size of the revealed protein bands was compared to the molecular weight ladder. β-actin was seen at its expected size of 42kDa and the chemokine domain of fractalkine was seen at its expected size of 8.5kDa.

2.8 Measurement of apoptosis

Induction of apoptosis in BL-cells

Irradiation with Ultraviolet Light UVB was routinely used to induce apoptosis in BL-cells. BL-cells in suspension were washed in fresh media $RPMI_{supp}$ and placed in flat-bottomed flasks and exposed to 200mJ/cm^2 of UVB in a transilluminator (UV products limited, UK). 16 hours after this treatment, routinely more than 90% of cells were apoptotic and trypan blue-positive. Alternatively, apoptosis was induced chemically by incubation with 1μM ionomycin for up to 16 hours, with 1μlml staurosporine for 8 hours, or 1μM actinomycin D for 8 hours. After chemical treatment cells were washed in fresh media and routinely, over 90% of cells were apoptotic and trypan blue-positive.

FACS-based measurement of apoptosis

The light scatter properties of viable and apoptotic BL-cells differ when analysed by FACS (Dive et al, 1992). Figure 2.8a(i) shows the forward versus side scatter plot of a mixture of viable and apoptotic BL-cells. The smaller and more-dense apoptotic cells fall close to the y-axis whereas the viable cells fall closer to the x-axis. For every experiment, the flow cytometric data was confirmed by analysis of nuclear morphology by staining of cytospins with H&E or checking nuclear morphology with 4,6-diamidino-2-phenylindole (DAPI) detailed below.

Annexin V and propidium iodide staining of BL-cells

All fluorescence labelling was carried out in the dark on ice. To measure apoptosis, 10^6 cells were washed and maintained in calcium containing buffer and labelled with annexin V (AxV) and propidium iodide (PI) (Bender Med Systems, USA). The AxV-binding buffer was made up as follows: 2ml of 10mM HEPES pH7.4, 9.3ml of a 0.1M stock of NaCl (140mM), 2.5ml of a 0.1M stock of $CaCl_2$ (25mM) made up to 100ml with distilled water. For each sample of cells, 10µl of PI (50µg/ml) 1µl FITC AxV (25µg/ml) and 89µl of buffer were added. After a 15 minutes incubation on ice in the dark, 400µl of binding buffer was added and run on the Coulter XL FACS machine.

4', 6-diamine-2'-phenylindole dihydrochloride (DAPI) nuclear staining

Cells were checked by light microscopy for phenotypic changes of apoptosis such as membrane blebing, pyknosis and cell shrinkage. Condensed chromatin was visualised by 4', 6-diamine-2'-phenylindole dihydrochloride (DAPI). Cells were first fixed in 1% formaldehyde for a minimum of 12 hours at 4C. DAPI was added to a final concentration of 0.25µg/ml. A drop of the cell suspension was placed on a glass slide (under a glass coverslip) and analysed by fluorescence microscopy (Axioskop2, Zeiss Switzerland). Two hundred cells per sample were scored as having viable or apoptotic morphology and the percentage of apoptotic cells was recorded for each experiment. Figure 2.8a(ii) shows that viable Mutu BL-cells have large round nuclei whereas, apoptotic cells have condensed and blebed nuclei with a typical "popcorn" appearance.

Transfection of BL-cells with *bcl-2*

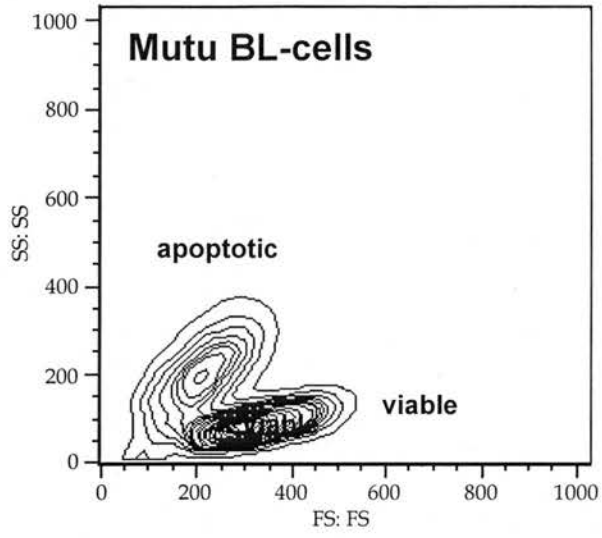
Once BL-cells had been transfected with *bcl-2*, they were resistant to the UVB-induction of apoptosis. 16 hours after induction of apoptosis 83% of Mutu BL-cells were in the apoptotic zone, but only 15% of the transfected cells were apoptotic (figure 2.8b). What is more, UVB-treated BL-cells transfected with *bcl-2* did not stimulate macrophage transmigration in the transwell assay. Therefore, UVB-treated-BL-cells (transfected with *bcl-2)* were not routinely used in experiments.

Preparation of necrotic cells

Mutu BL-cells were placed in fresh $RPMI_{supp}$ in the absence of serum at a concentration of 10^6/ml. Cells were then placed in a water bath at 100C and boiled for 20 min. All cells were trypan blue-positive after this treatment.

The measurement of BL-cell apoptosis

i



ii

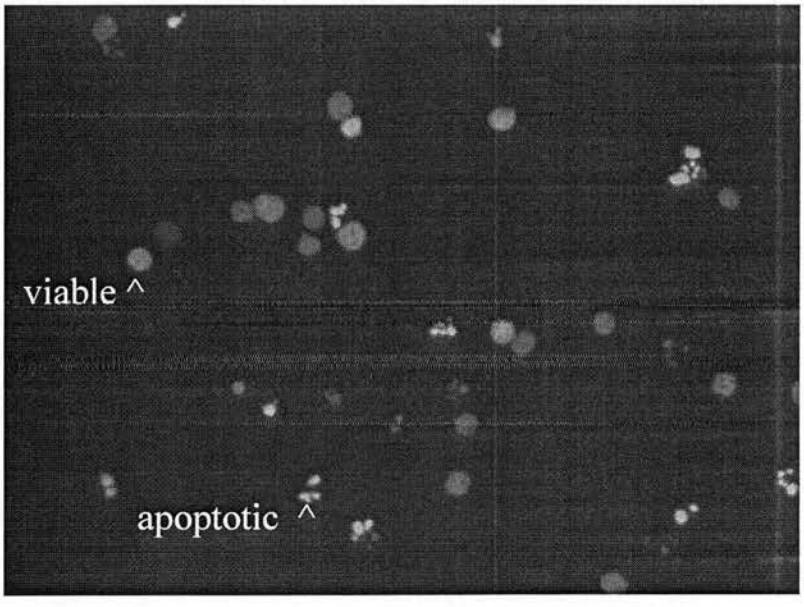


Figure 2.8a
(i) FACS light scatter plots of BL-cells showing 'viable' and apoptotic' zones. (ii) DAPI nuclear labelling of viable cells with large round nuclei and apoptotic BL-cells that have condensed nuclei and a "popcorn-like" appearance.

BL-cells transfected with *bcl-2* are more resistant to apoptosis

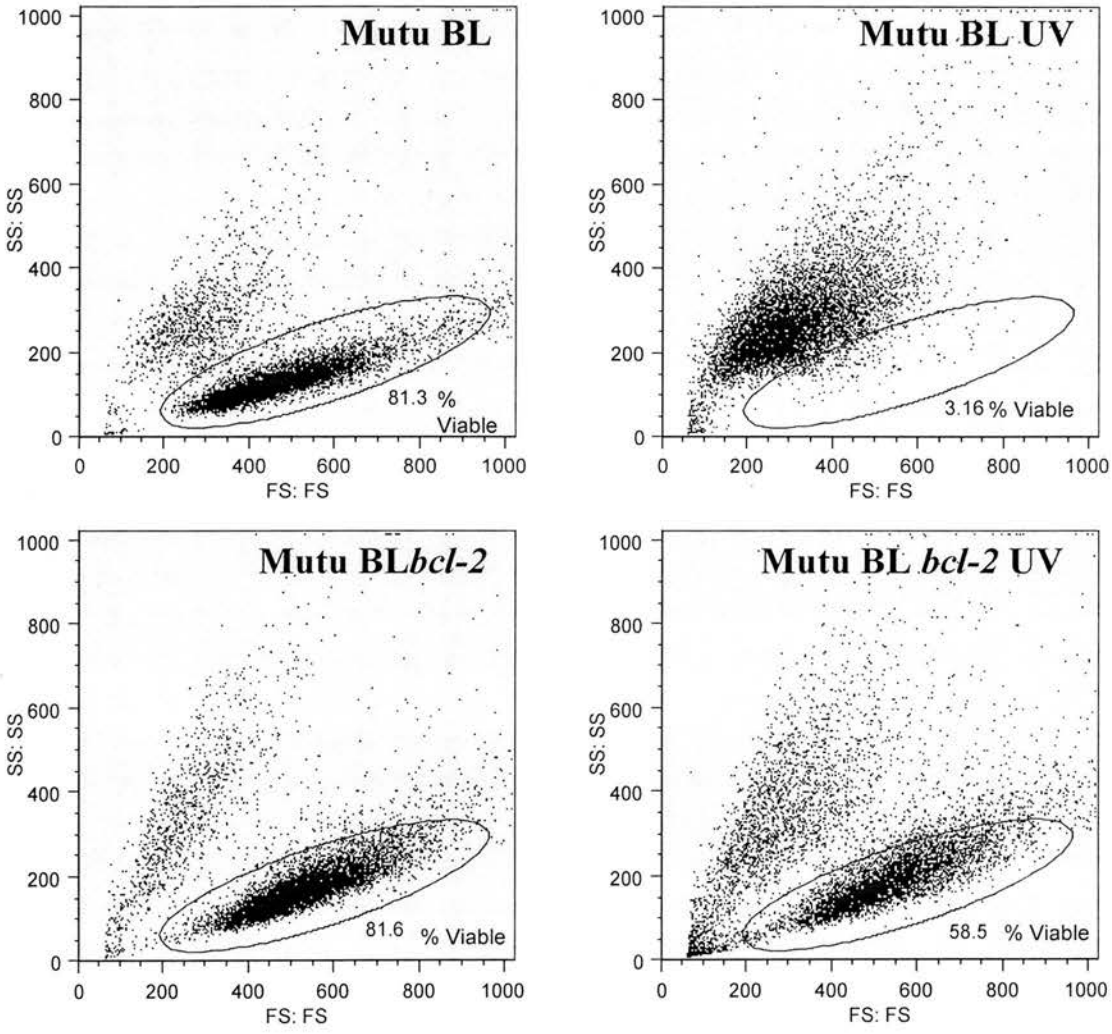


Figure 2.8b

FACS analysis of light-scatter properties of Mutu. Forward scatter (FS) versus side scatter (SS) is plotted. Mutu with and without *bcl-2* were examined 16 hours after UV-induction of apoptosis. Mutu are shown falling into the viable zone. Only 3.16% of Mutu BL fall in the viable zone after UV-treatment . Mutu *bcl-2* are relatively resistant to apoptosis and have more viable cells after UV-treatment (58.5%).

2.9 Macrophage-apoptotic cell interaction assay

Human monocyte-derived macrophages and murine bone marrow-derived macrophages (BMDM) were seeded into 48 well plates. Ten-day old macrophages were used for all phagocytosis studies. Mutu were induced to become apoptotic by 100mJ/cm^2 UVB-light.16 hours after UV treatment over 90% of the Mutu were apoptotic. Apoptosis was confirmed by the ability of cells to bind annexin-V FITC and by DAPI staining of alterations in the nuclear morphology of the apoptotic cells. Apoptotic cells were twice washed in sterile PBS and resuspended in serum-free $RPMI_{supp}$ at a concentration of $5x10^6$ cell/ml.

Macrophages were washed and placed in serum-free media $DMEM_{supp}$ for thirty minutes. Media was aspirated and the wells were washed with PBS. The suspension of apoptotic cells was added to the wells at a ratio of five apoptotic Mutu to one macrophage. The cells were co-incubated for 0-75 minutes at 37C and 5% CO_2. Each condition was set up in triplicate. Control wells containing only macrophages without apoptotic cells, measured the baseline level of macrophage phagocytosis. At the end of the experiment, wells were vigorously washed with PBS to remove any non-adherent cells. The plate was viewed under the light microscope to ensure that apoptotic cells had been thoroughly removed before fixing in 1% formalin overnight at 4C. Plates were washed with PBS and stained with haematoxylin (DiffQuik II Dade) for five minutes and then washed once more in PBS. The nuclei of the macrophages and the shrunken pyknotic nuclei of the apoptotic cells inside the macrophages was seen clearly at x400 magnification using an inverted microscope. Two hundred macrophages were counted in each well and the percentage of macrophages that had interacted with apoptotic cells was calculated. The mean and standard deviation of the mean was calculated for three wells.

2.10 Macrophage transmigration assay

Introduction

This thesis examines macrophage migration to human and murine apoptotic cells. Primary macrophages are large, adherent and terminally differentiated cells

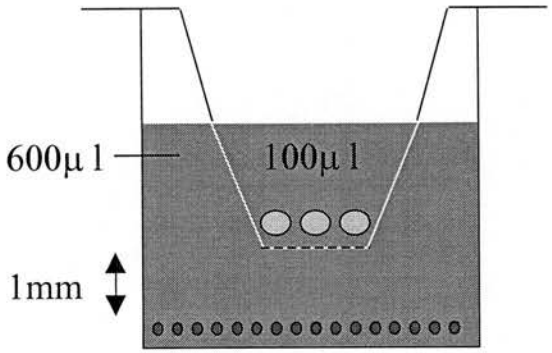
that are not often studied in chemotaxis assays *in vitro*. Instead, monocyte cell lines are usually substituted for primary macrophages. More mobile monocyte cell lines can be transfected with chemotactic receptors and used as surrogate macrophages. There are many ways of measuring chemotaxis: Sophisticated time-lapse digital imaging can be used to measure macrophage directional movement both *in vitro* and *in vivo*. Monocyte and macrophage migration can be monitored under agarose but this requires very accurate measuring of the "front" of migrating cells because they might only move 1-2mm in the space of 4 hours (Heit, 2003). An alternative is to wound a macrophage monolayer by the scraping off of a line of adherent macrophages from the bottom of a tissue culture dish; this allows the movement of neighbouring cells, as they move into the wound site, to be monitored.

The transwell assay is a simple device for measuring cell chemotaxis. Cells are placed in an upper chamber and are allowed to transmigrate through a porous membrane towards chemotactic molecules. The setting up of a robust transwell system to measure macrophage chemotaxis to apoptotic cells is described in this chapter.

Preparation of cells to be placed in the lower chamber

BL were passaged 48 hours prior to use in the assay. 10-30ml of BL were placed under a UVB transilluminator (UV products limited, UK) and exposed to 200mJ/cm^2 of UVB light (approximately 20 minutes) at RT. 12 hours after UV-induction of apoptosis BL, were washed in RPMI $_{supp}$ without serum, centrifuged at 1000 RPM and resuspended serum-free RPMI$_{supp}$ at a concentration of 2x10^6 cells/ml. BL$_{bcl\text{-}2}$ or untreated BL-cells were washed and resuspended to a concentration of 2x10^6 cells/ml in the same way. 600µl of each cell suspension or control solution was pipetted into a separate well of a 24-well tissue culture plate (Costar, UK) and kept at 37C and 5%CO_2. In addition, 600ul of serum-free RPMI$_{supp}$ was used as a negative control. Either 10^{-8}M fMLP (*N*-formylated-Methionine-Leucine-Phenylalanine) (Sigma, UK) or recombinant CCL5 (Peprotech, UK) was diluted in 600ul of serum-free RPMI$_{supp}$ to a final concentration of 100ng/ml and was used for positive controls. All conditions were set up in duplicate. A separate well of 600µl of cells (one for each condition) was set up that was not used in a chemotaxis

Transmigration assay

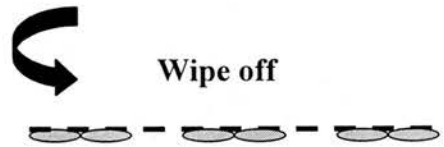


Macrophage

Mutu BL-cells

(i) 10^5 Macrophages placed on a 5µm pore-filter above $2x10^6$/ml Mutu BL-cells

(ii) Macrophages allowed to migrate at 37°C for 4 hours



(iii) Macrophages scraped off the upper side of filter

(iv) Migrated macrophages fixed in methanol, stained with haematoxylin and the number of transmigrated cell counted in ten random fields viewed at high-power

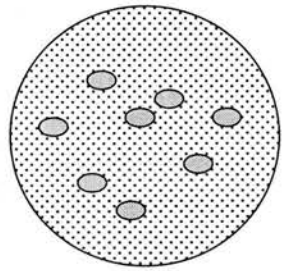


Figure 2.10
A schematic diagram showing the protocol of the transmigration assay used to measure macrophage chemotaxis to apoptotic cells

assay. Instead, cells in these wells were used to assess the number of apoptotic cells present in each well at the end of the experiment. FACS was used to assess apoptosis using Annexin V and PI labelling, and nuclear morphology was assessed by microscopy of DAPI stained cells. Once all conditions had been set up, the plate was returned to the 37C incubator whilst the macrophages (or other migrating cells) were prepared for use.

Preparation of cells to be placed in the upper chamber

Primary eight day-old macrophages were grown in 6-well tissue culture plates (Costar, UK). Non-adherent cells were removed by washing with cold, sterile PBS (4C). 1ml of cold PBS was pipetted over the macrophages and plates were placed on ice for 5 minutes. Macrophages were harvested using a cell lifter (Costar, UK), washed in $RPMI_{supp}$ without serum, centrifuged at 1000rpm and resuspended at a concentration of 10^6 macrophages/ml. The number of cells was counted on a haemocytometer and viability was assessed using trypan blue exclusion. Further manipulations such as the incubation with antibody for 30 minutes, or the addition of chemokines and inhibitors were done at this point.

Chemotaxis experiments were performed in 6.5mm diameter transwells (Costar,UK) with an uncoated polycarbonate membrane containing 5μm-diameter pores, using a modification of Boyden's technique (Barleon, 1996). 10^5 macrophages/monocytes or neutrophils were placed in 100μl of $RPMI_{supp}$ in the top of a transwell and allowed to transmigrate to the bottom well at 37°C, 5% CO_2 for either 1 hour (neutrophils) or 4 hours (monocytes and macrophages). Transwell inserts were removed and non-migrated cells were removed from the upper well by pipette. The upper membrane was wiped using a clean cotton bud. The lower side of the filter was not disturbed as the migrated cells were adherent to the underside. Filters were fixed in 100% methanol for ten minutes and allowed to dry and each transwell was labelled with a permanent marker pen for identification. Cells on the filter were stained by emersion into a well containing Dif-Quik II (Dade, Germany) for ten minutes. Transwells were tapped dry and placed into a clean 24-well plate. The number of cells that had migrated was counted in 10 random high-power fields (x400 magnification) using an inverted microscope (Zeiss Axiovert 25). The mean

number of cells counted in ten high power fields was calculated and significance was measured using the Student's t-test. Experiments were repeated a minimum of three times. All data was graphed and analysed using Graph Pad Prism software version 3.02.

In one experiment, the macrophages that had passed through the filter membrane, towards apoptotic BL-cells were studied. At the end of the transmigration assay, the non-migrated cells were removed from the upper well in the usual way and immunolabelled with anti-CD14 antibodies and analysed by FACS. Placing the filters in ice-cold 2mM EDTA isolated the macrophages that had migrated to the lower surface of the filter. These adherent macrophages were freed by gently tapping the inserts. A minimum of ten filters was required to harvest enough macrophages for analysis. After washing in PBS, the CD14 expression of the macrophages that had migrated to apoptotic BL-cells was compared to non-migrating cells in the upper well.

Preparation of supernatants

BL-cells were washed and placed in serum-free media and treated with UVB-light to induce apoptosis and kept in an incubator at 37C. 16 hours later over 90% of cells were apoptotic. Cells were gently spun down at 1200 RPM supernatants were aspirated off and passed through a 0.2μm-pore filter. Likewise, supernatants were collected from live BL-cells and BL-cells transfected with *bcl-2* that had not been exposed to UVB. After boiling at 100C for 20 minutes, necrotic-cell supernatants were prepared also passed through a 0.2μm-pore filter.

Controls

Each transwell experiment included a negative control (media only in the lower chamber and a positive control (either CCL5 or fMLP in the lower chamber. In addition every experiment included wells containing 2x $10^{6/ml}$ BL-cells for the purpose of monitoring the rate of cell apoptosis that varied between experiments. Chemotaxis is directional cell motion along a concentration gradient. Chemokinesis is the increased activity (including movement) of a cell stimulated by a chemical. Chemokinesis itself may increase the number of cells passing through the filter. To test whether apoptotic BL-cells were truly chemotactic to macrophages, the same

concentration of cells was placed above and below the filter. There was no concentration gradient across the filter in these chemokinesis-control wells. The number of macrophages stimulated to move by chemokinesis was subtracted from the number responding to the chemotactic gradient. A checkerboard analysis was done to establish whether apoptotic BL-cells and their supernatants were true chemokines. Increasing concentrations of the BL-cells and their supernatants were placed in the upper chamber to abolish the chemotactic gradient. Apoptotic BL-cells and their supernatants were judged to contain true chemokines because increasing the concentration in the upper chamber reduced macrophage migration through the filter. If apoptotic BL-cells had simply caused chemokinesis, macrophage migration would have been simulated by the overall increase in BL-cell concentration.

Inhibition of transmigration by vMIPII, CX3CL1 and pertussis toxin

The anti-fractalkine monoclonal antibody clone 51637.11 (R&D systems) was titrated for its ability to block macrophage migration to the 8kDa chemokine domain of fractalkine (Peprotech) in a transmigration assay. The ND_{50} dose for this antibody quoted in the data sheet was 15-30μg/ml. The number of macrophages that transmigrated to the chemokine domain of fractalkine was much lower than responded to apoptotic BL-cells. However, a significant reduction in macrophage chemotaxis of more than 50% was observed when 50μg/ml of antibody was placed in the lower chamber with the apoptotic BL-cells.

The viral peptide vMIPII (macrophage inflammatory protein II) was also titrated for its ability to block macrophage migration to the 8.5kDa chemokine domain of fractalkine (Peprotech) in a transmigration assay (Boshoff, 1997). Again, macrophage transmigration to CX3CL1 was of a lower magnitude than to apoptotic BL-cells. A concentration of 300ng/ml vMIPII was able to significantly block macrophage transmigration and was used to block macrophage transmigration to apoptotic BL-cells.

Pertussis toxin (PTX) inactivates the G_i subunit of the trimeric G-proteins that are activated by chemokine receptors, including CX3CR1. Macrophages were pre-treated with 100ng/ml of PTX (Alexis) overnight at 37C. Cells were washed well before use in transmigration assays. There was no difference in macrophage viability as judged by trypan blue exclusion between the PTX-treated and untreated cells.

Adaptation of the transmigration assay for Mono mac 6 cells

Mono mac cells (MM6) are a monocyte cell line. When MM6 were substituted for macrophages in the transmigration assay, they did not adhere to the underside of the filter membrane but passed directly into the lower chamber. Counting the number of transmigrated MM6 was performed most reliably and reproducibly done by FACS. BL-cells were first stained with a Calcein AM fluorescent dye (Molecular probes) in order discriminate between the migrated MM6 cells and apoptotic BL-cells. Mutu BL-cells were labelled fluorescent green using Calcein AM using the following protocol. Mutu BL-cells were washed and resuspended in RPMI at a concentration of $2x10^7$cells/ml. 1ul of Calcein AM green (from a 1mM stock) was added per ml of cells to a final concentration of 1μM and incubated at 37C for 15 minutes. Cells were then thoroughly washed in RPMI and resuspended at 10^6cells/ml for induction of apoptosis to be used in the transmigration assay. After the assay the transwell inserts were removed and 400μl of RPMI containing 2Mm EDTA was added to the lower chamber. Cells were pipetted from wells and vortexed before counting on the FACS machine. BL-cells were gated out by both size and fluorescence. The reproducibility of counts was measured by the addition of a known number of flow-calibration beads (Coulter). Three, 30-second counts were made from each well. Each experiment was performed in duplicate wells and the average number of MM6 cells that migrated was calculated from six counts.

Adaptation of the transmigration assay for mouse BMDM

Bone marrow derived murine macrophages were used after 10-14 days in culture. The protocol for measuring mouse macrophage transmigration to human BL-cells was changed so that 10^{-8}M fMLP was used as a positive control in place of human CCL5. BMDM were washed in 0.01% BSA to prevent clumping of cells and adherence to tissue culture plastic. In addition, Both the BL-cells and BMDM were suspended in media containing 0.01% BSA for the transmigration assay.

Chemicals and reagents

All chemicals were supplied by Sigma unless indicated otherwise in the text or listed below:

Acetic acid	BDH chemicals
Acrylamide	Biorad
Annexin V (FITC conjugated)	Biowhittaker
Biorad Protein assay	Biorad
Diff Quik	Dade
DePeX mounting medium	BDH chemicals
ELC reagents	Amersham Biosciences
Ethanol	BDH chemicals
EDTA	Fischer
Formaldehyde	BDH chemicals
Glycine	Fischer
Hydrochloric acid	Fischer
Ionomycin	Calbiochem
Mowiol	Calbiochem
Non-fat skimmed milk powder	Tesco
Percoll	Amersham Biosciences
Propidium Iodide	Bender Medsystems
PVDF membrane	Amersham biosciences
Sodium Chloride	Fischer
Sodium Hydroxide	Fischer
Sodium phosphate dibasic anhydrous	Fischer
Sodium phosphate monobasic anhydrous	Fischer
Tris	BDH chemicals
Trisma base	Fischer
Triton-X-100	Biorad

Cell culture

DMEM Dulbeco's modified eagle medium	Gibco
FBS foetal bovine serum	Biowhittaker
G418 sulphate	Gibco
IMDM Iscoves modified Dulbecco's medium	Gibco
L-glutamine	Gibco
Macs beads	Milteny Biotech
Non essential amino acids	Gibco
Normal goat serum	Harlan Sera-labs
Penicillin-streptomycin	Gibco
RPMI 1640 medium	Gibco
Serum supreme	Biowhittaker

Molecular biology

10mM dNTP solution	Invitrogen
DNA 100bp ladder	Invitrogen
DNA 1Kb ladder	Invitrogen
DNA oligonucleiotide primers	MWG-Biotech
Dnase	Invitrogen
Random hexamers	MWG-Biotech
Reverse transcriptase	Promega
RNAse A	Promega
Taq polymerase	Roche

Equipment

Centrifuges

Beckman GS-6R	Beckman
Beckman J2-HS with JA14 and JA21 rotors	Beckman
Rotina 46-R	Hettich

Sigma 1-15K	Sigma

Cell culture

Auto MACS	Milteny Biotech
BS7 48 neubauer haemocytometer	Weber
Conical polypropylene tubes 15 and 50 ml	BD Bioscineces
Incubator	LEC
Microflow class II hood	Walker safety cabinets
Tissue culture plastics	Nunc
Transwells 6.5 diameter	Costar

Microscopes

Fluorescence microscope Axioskop 2	Zeiss
Leica inverted TCS-NT microscope	Leica Microsystems
Leica upright TCS-NT microscope	Leica Microsystems
Olympus CK2 light microscope	Olympus
TCS-NT laser scanning unit	Leica Microsystems

General

800W microwave	Proline
Anthos It-2 plate reader	Anthos labtech instruments
Cecil-200 spectrophotometer	Cecil instruments
Coulter EPICS XL-MCL FACS machine	Beckman Coulter
Waterbath	Grant Instruments
Horizon gel tanks	Life technologies
Mini protean II gel system	Biorad
Transilluminator	UV products Ltd

Antibodies

NAME	*CLONE*	*SPECIES*	*CLASS*	*LABEL*	*MANUFACTURER*
CX3CR1	polyclonal	Rabbit	IgG		MBL
CX3CL1	polyclonal	Goat	IgG		R&D systems
CX3CL1	81506	Mouse	IgG1		R&D systems
CX3CL1	polyclonal	Goat	IgG	Biotin	R&D systems
CX3CL1	51637	Mouse	IgG1		R&D systems
CX3CL1	81513	Mouse	IgG1		R&D systems
CD14	TuK4	Mouse	IgG2a	RPE	Serotec
CD14	UCHM1	Mouse	IgG2a	RPE	Serotec
CD14	TuK4	Mouse	IgG2a	RPE	Dako
CD14	61D3	Mouse	IgG1		Birmingham University
CD14	63D3	Mouse	IgG1		Birmingham University
CD68	PG-M1	Mouse	IgG3		Dako
Isotype control		Mouse	IgG1		Sigma
Isotype control		Mouse	IgG2a		Sigma
Isotype control		Mouse	IgG3		Sigma
Isotype control	MCA 929	Mouse	IgG2a	RPE	Serotec
Isotype control	MCA928	Mouse	IgG1	RPE	Serotec
Anti-mouse Fab	polyclonal	Goat	Ig Fab	RPE	Dako
Anti-mouse Ig	polyclonal	Goat		HRP	Dako
Alexa Flour 488	A-11001	Goat	Ig Fab	488	Molecular probes
Anti-mouse Ig	polyclonal	Goat		FITC	
Anti –mouse Ig	polyclonal	Goat		HRP	

CHAPTER 3

The role of CD14 in the clearance of apoptotic cells

Introduction

This laboratory established an important role for CD14 in the clearance of dying cells, by showing that antibodies against CD14 could reduce macrophage phagocytosis of apoptotic cells *in vitro* (Devitt, 1998). When the CD14 knockout mouse (CD14-/-) was produced some years ago using a gene targeting approach, it became possible to investigate the role CD14 *in vivo* (Haziot, 1996). Detailed histological studies showed that a number of organs in the Balb/c CD14-/- contained persistent apoptotic cells compared to wild type mice (Devitt, 2004). These apoptotic cells were described as "free" because they were not associated with macrophages, suggesting that the accumulation of apoptotic cells might be due to a defect in macrophage clearance, especially since both the knockout and the wild type mice had equivalent numbers of tissue macrophages. Since the rates of apoptosis were also comparable between the knockout and wild type mice, this raised the possibility that there was a defect in macrophage clearance of apoptotic cells in the Balb/C CD14-/- that resulted in the persistence of cell corpses observed *in vivo*. Apoptotic cells that were injected directly into the peritoneum of wild type mice were swiftly phagocytosed (in about 15 minutes) but this was significantly delayed in mice without CD14 (Devitt, 2004). Again this suggested that CD14 was important for the process of clearing away dying c ells *in vivo* (Devitt, 2004).

The experiments presented in this chapter sought to confirm published work and to extend these observations to the C57BL/6 mouse strain. The thymus of CD14 knockout (CD14-/-) mouse was studied and the number of apoptotic cells in the organ was compared to the wild type. Because the CD14-/- macrophages have a defect in the tethering of apoptotic cells to macrophages, it was hypothesised that the CD14-/- would have increased numbers of apoptotic cells *in vivo*.

The persistence of apoptotic cells in the CD14-/- thymus was examined on both the Balb/c and the C57BL/6 backgrounds, so as to reveal any specific differences in between the two mouse strains. The Balb/c mouse has a polarised T-helper cell type-2 response (TH2) resulting in secretion of IL-4 and TGFβ in response to overwhelming Leishmania infection. In contrast, C57BL/6 strains clear this pathogen through a TH1 response by secreting IFN-γ (Heinzel, 1989). Generally, macrophages have more flexibility in their function compared to lymphocytes, and

similarly, they can be skewed to either a M1 or a M2 phenotype (Gordon, 2003). In the presence of TGFβ, an "alternatively activated" or M2, macrophage may have reduced nitric oxide production and killing but improved phagocytosis (Mills, 2000). Considering the polarisation of these two strains, a different ability to clear away of apoptotic cells between the Balb/c and the C57BL/6 mice might be predicted.

Finally, a population of $CD14^{low}$ macrophages were isolated from human blood and their ability to phagocytose apoptotic cells was compared to the $CD14^{high}$ subset of monocyte-derived macrophages (Ziegler-Heitbrock, 1996).

3.1 The Balb/c CD14-/-mouse-thymus contained an increased number of apoptotic cells compared to the wild type

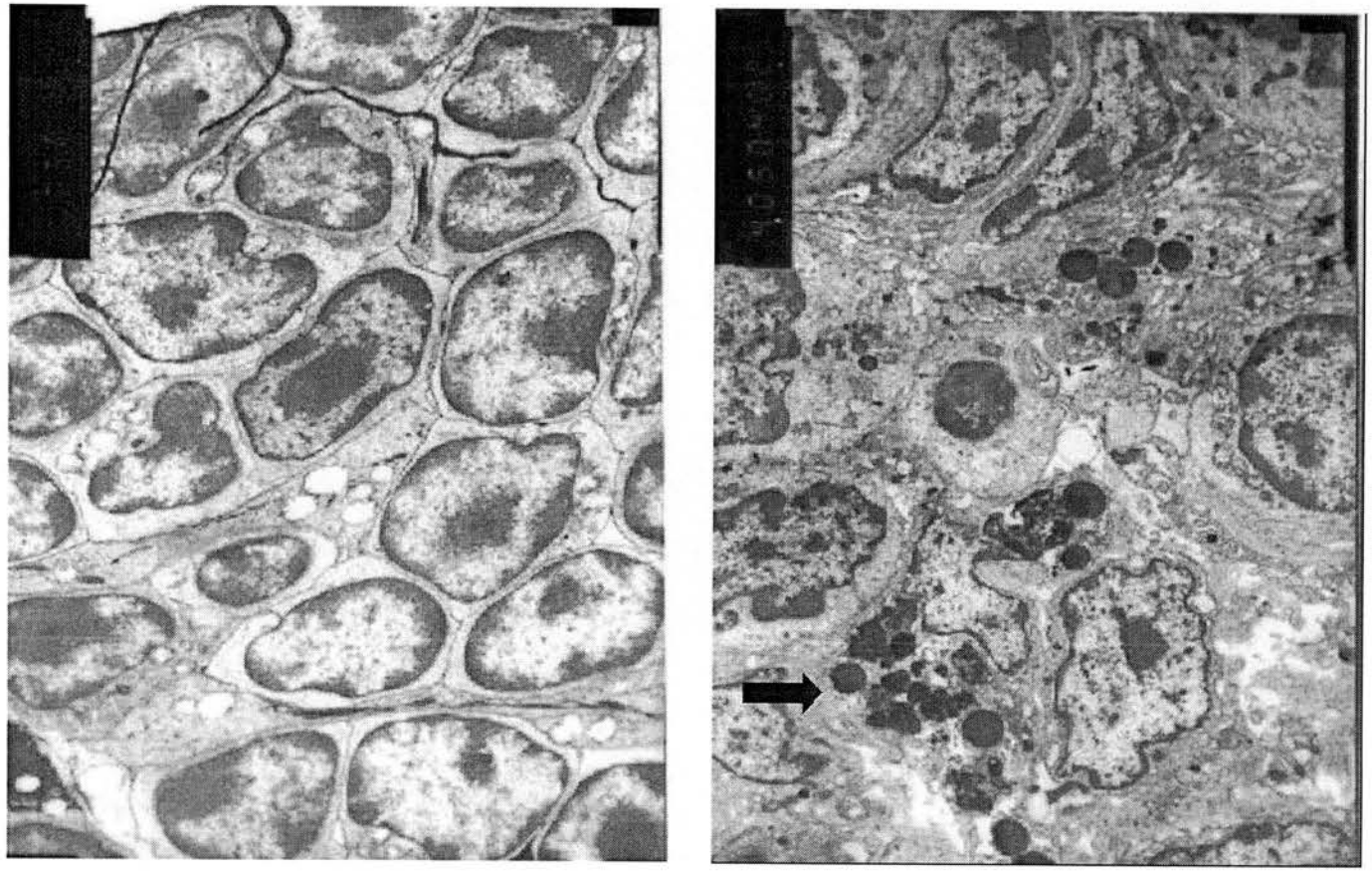
The thymus of a young adult mouse is the site of T cell selection and normally contains large numbers of apoptotic thymocytes. Treatment with a steroid like dexamethasone, 24 hours prior to sacrifice, can be used to induce a synchronous and dramatic apoptosis of thymocytes (Wyllie, 1982; Devitt, 2004). Transmission Electron Microscopy (TEM) was used to visualise thymus tissues from the CD14-/- mouse *ex vivo* and the number of apoptotic cells was qualitatively examined.

Balb/c CD14-/- thymus observed by Transmission Electron Microscopy (TEM)

(Figures 3.1a)

Each thymus was briefly examined before processing for TEM. There was no gross evidence of infection or tumour present in the thymi examined from either the Balb/C CD14-/- or from the wild type, and the wet weight of the thymi was similar. The thymi from the mice that had been treated with 200μg of dexamethasone were smaller than the mock-treated (PBS) control organs.

Increased numbers of apoptotic cells in the Balb/c CD14-/- thymus



i) CD14+/+ wild type **ii) CD14-/-**

Figure 3.1a

Transmission electron microscopy (TEM) of i) wild type thymus and ii)CD14-/- . CD14 deficient thymus contained increased numbers of apoptotic cells (arrow).

The thymi from CD14-/- and wild type were examined under the electron microscope and there were more apoptotic cells in the thymi from the knockout mice. It was common to see more than two apoptotic cells per visual field (at 2000x magnification) in the CD14 knockout, and the tissue macrophages were observed to contain apoptotic bodies.

The thymi from CD14-/- and wild type mice that were treated with dexamethasone, or a mock PBS injection, were next examined under the electron microscope (*figure 3.1a)*. Mice that lacked CD14 had more apoptotic cells than the wild types. Dexamethasone induced a massive amount of apoptosis in both mice, and again, the CD14-/- thymus contained more apoptotic cells than the wild type. In sections of the dexamethasone-treated CD14-/- thymus, it was usual to observe at least three apoptotic cells per visual field (at 2000x magnification).

Overall, there was a qualitative increase in the number of apoptotic cells in the CD14-/- compared to the wild type thymus. Objective measurement of the number of apoptotic cells in each thymus was not possible because of the small numbers of animals used in each group and the limited access to the TEM. Nevertheless, the effect of dexamethasone was apparent in both the wild type and knockout animals both macro and microscopically.

Increased numbers of Annexin V-positive thymocytes in Balb/c CD14-/- *ex vivo*

(Figure 3.1b)

Annexin V (AxV), which binds to exposed phosphatidylserine on apoptotic cells was used to label dying cells. FACS analysis of AxV-positive cells can be used to quantify the number apoptotic cells within an organ *ex-vivo* (Dive, 1992; Koopman, 1994). The FACS data from this experiment is shown in Figure 3.1c. Injection with dexamethasone increased the number of Annexin V-positive cells in both the CD14-/- and the wild type thymus, but overall, the level of apoptotic cells was always higher in the CD14-/-.

Increased numbers of Annexin-V positive apoptotic cells in the Balb/c CD14-/- thymus

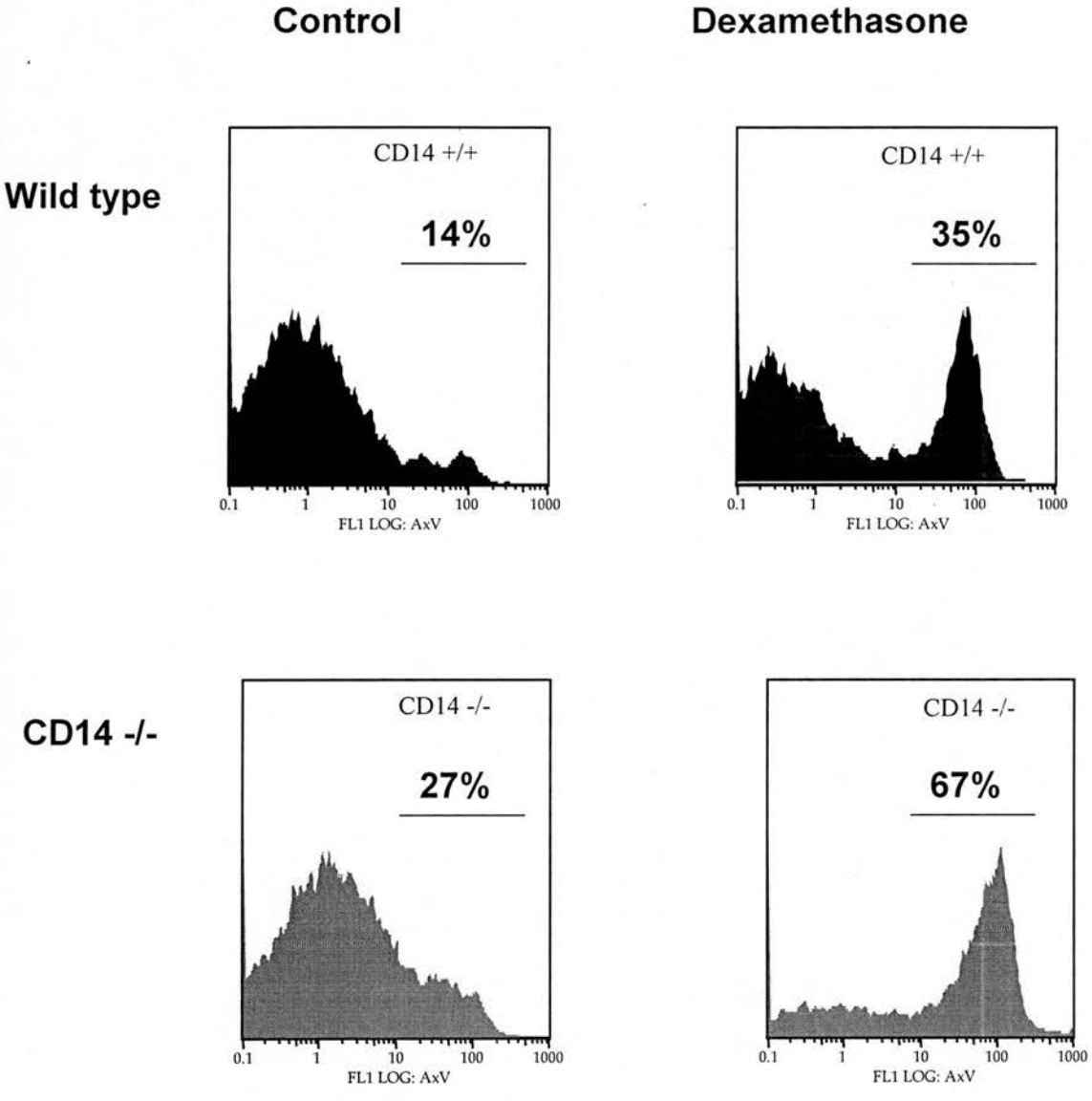


Figure 3.1b

Flow cytometry of thymocytes from CD14-/- and wild type mice treated with PBS (control) or 200μg dexamethasone. The log-fluorescence intensity of FITC-labelled AxV in CD14+/+ wild type (black) and CD14-/- (grey) thymocytes. The percentage of cells falling into the apoptotic gate are labelled.

Increased numbers of TUNEL-positive cells in the Balb/c CD14-/- thymus

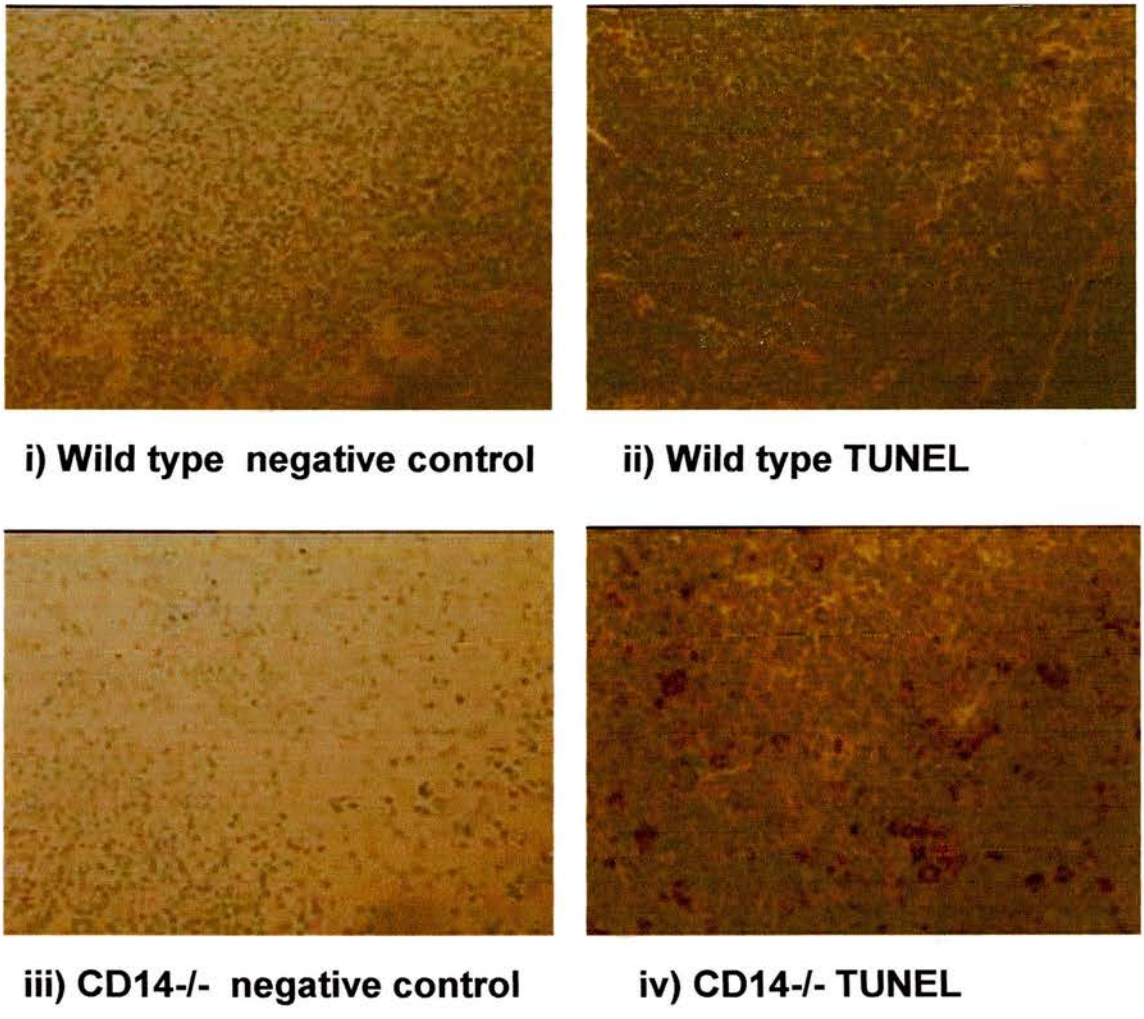


Figure 3.1c
TUNEL-staining of apoptotic cells (brown) in the thymus of Balb/c CD14-/- (iv) and wild type cells (ii) compared to a negative controls. Methyl green counter-stain.

CD14-/- thymus shows increased numbers of TUNEL-positive apoptotic thymocytes

(Figure 3.1c)

During apoptosis, a cell cleaves its DNA into small fragments. It is these different-sized lengths of DNA that give rise to the characteristic "ladder" appearance when DNA is run out on a gel. TUNEL (TdT-dependant dUTP-biotin nick end labelling) is a biochemical method of identifying apoptotic cells in tissue. A Biotin-tagged dUTP is added to the free 3' ends of the DNA fragments by a TdT enzyme. Subsequently the fragmented DNA can be visualised by streptavidin linked to a peroxidase. Finally a (brown) colour substrate is added in order to visualise the free "nicked ends" of fragmented DNA in apoptotic cells.

TUNEL staining confirmed the earlier TEM and FACS findings by showing increased numbers of apoptotic cells in the thymus of the Balb/c CD14-/- mice compared to wild type. Figure 3.1d is a representative image showing both cortex and medullary regions of the thymus at 200x magnification. Apoptotic cells were stained brown and all nuclei were counterstained green. The majority of apoptotic cells were found in the cortex, close to the cortico-medullary junction.

Three different methods, TEM, FACS and TUNEL-labelling, were three methods used to show that the Balb/c CD14-/- had persistent apoptotic cells in its thymus. The qualitative results from this small preliminary experiment are in agreement with previous work (Devitt, 2004).

It was conceivable that CD14-/- cells might be more sensitive to dexamethasone than wild type thymocytes and the high numbers of apoptotic cells in the CD14 knockout mouse were due to an increase in the cell death rate. However, earlier work had shown that thymocytes from the CD14-/- die at exactly the same rate as the wild type thymocytes after *ex vivo* steroid treatment (Devitt, 2004).

3.2 Quantitative analysis of the numbers of apoptotic cells in the thymus of the CD14-/- mouse

(Figures 3.2a and 3.2b)

TUNEL staining was used to quantify the number of apoptotic cells in thymi of Balb/c CD14-/- mice compared to wild type animals. Thymus tissues were

examined from six mice in each group. Organs were fixed in formalin, embedded in wax and sectioned. Representative sections from the full thickness of the thymus were mounted onto glass slides. After TUNEL-labelling, the number of apoptotic cells in the cortex and medulla was enumerated.

The CD14-/- BALB/c thymic cortex has increased numbers of TUNEL-positive cells compared to that of the wild type animals

(Figure 3.2a)

There was a significant increase in the number of TUNEL positive cells in the cortex of the BALB/c CD14-/- mice compared to wild type (Student's t-test $**p<0.005$). This result was in accordance with previous observations from this laboratory (Devitt, 2004). There was a small increase in the number of TUNEL positive cells in the medulla of the BALB/c CD14-/- mouse compared to wild type controls. However, it did not reach statistical significance.

Balb/c CD14-/- bone-marrow derived macrophages have impaired interaction with apoptotic cells *in vitro*

(Figure3.2b)

Having shown that CD14-/- Balb/c mice had increased numbers of apoptotic cells in their thymi, bone marrow derived macrophages (BMDM) were studied *in vitro*. It was hypothesised that apoptotic cells accumulated in Balb/c CD14-/- because CD14-/- macrophages were defective in their ability to clear away apoptotic cells. The ability of macrophages that lacked CD14 to clear away apoptotic lymphocytes was investigated using a standard interaction assay.

Cells from the Burkitt's lymphoma cell line "Mutu" were treated with UV-light to induce apoptosis (Gregory, 1990). These cells were then "fed" to a monolayer of BMDM in culture and the number of phagocytosed cells was counted. BMDM from CD14 knockout mice was compared to wild type macrophages. The phagocytosis assay was performed in serum-free conditions and apoptotic cells were incubated with macrophages, for 15, 30, 45, 60 or 75 minutes.

Balb/c CD14-/- macrophages phagocytosed fewer apoptotic Mutu BL-cells compared to wild type macrophages. After a 1-hour incubation, fewer CD14-/- macrophages interacted with apoptotic cells compared to the wild type ($*p<0.05$)

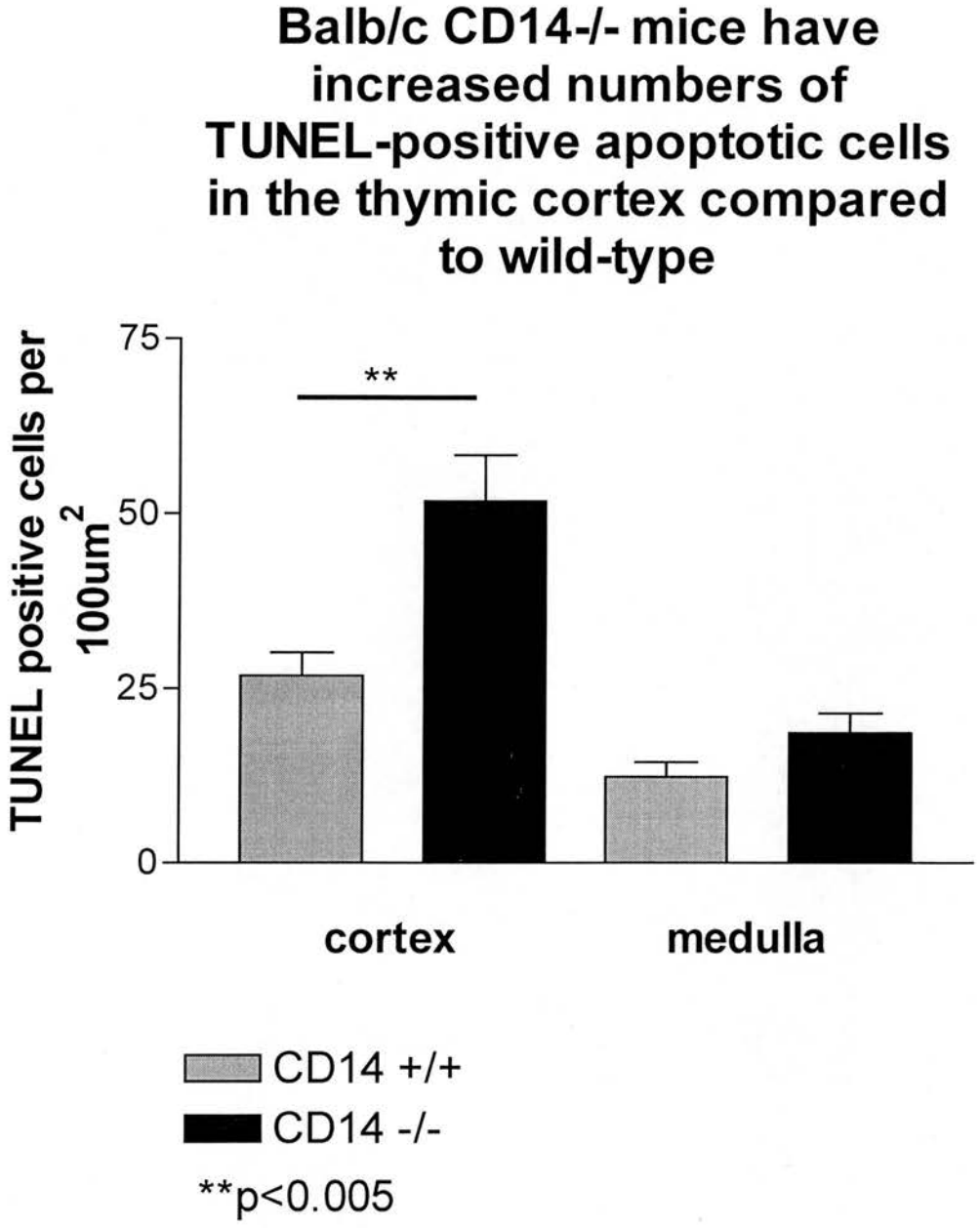


Figure 3.2a

The number TUNEL-positive cells in the medulla and cortex of thymus from Balb/c CD14-/- (black) mice (n=6) compared to wild type (grey) (n=6).CD14-/- have significantly higher numbers of apoptotic cells in the thymic cortex than the wild type ($p<0.005$, Student's t-test).

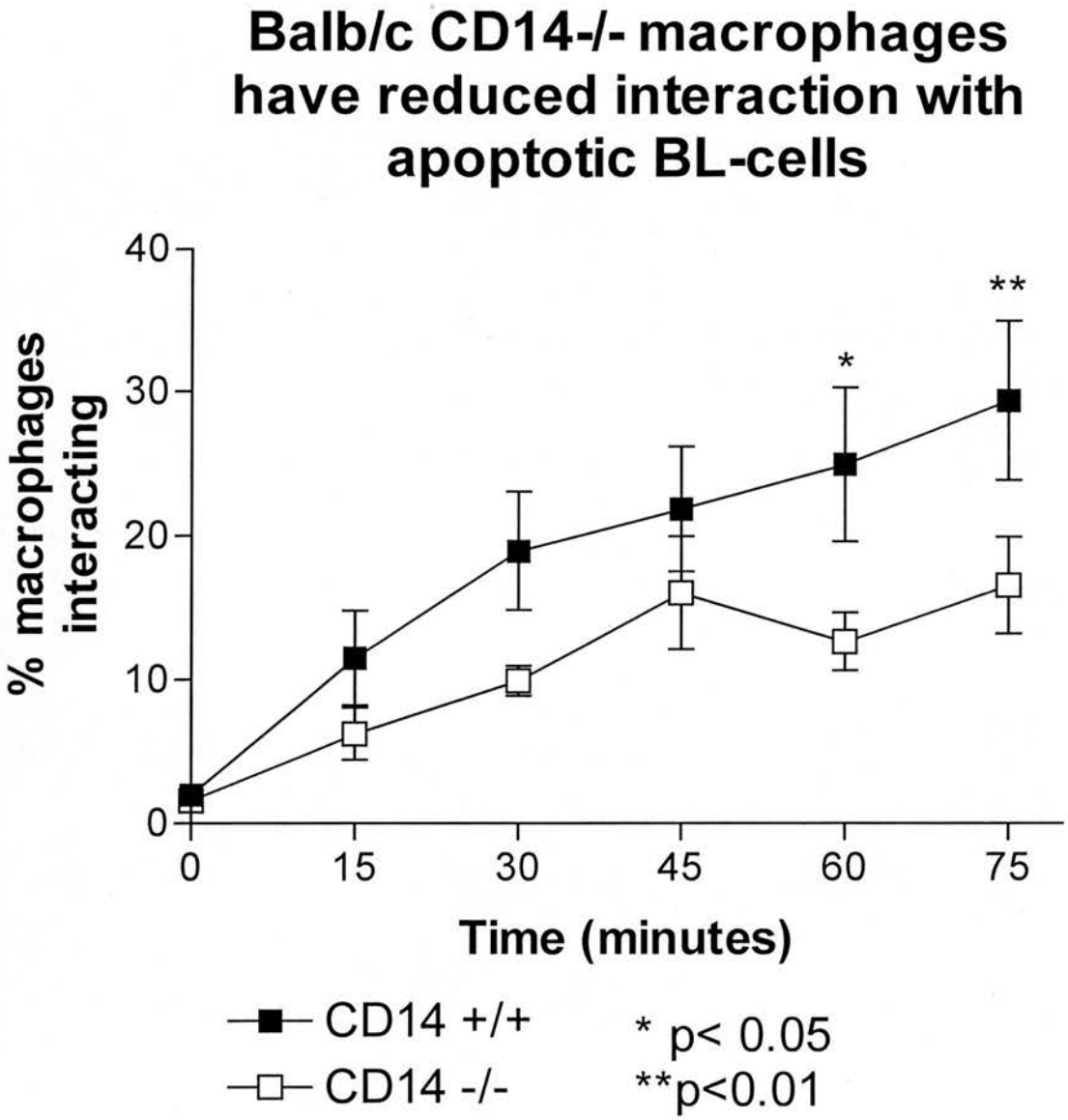


Figure 3.2b

CD14-/- bone marrow-derived macrophages (BMDM) (white squares have reduced interaction with apoptotic Mutu BL-cells compared to wild type macrophages (black squares). The reduction in phagocytosis was significant after 60 minutes. (*p<0.05, Student's t-test).

(figure 3.2b). The lack of CD14 did not have a significant effect on the clearance of apoptotic cells at earlier time points.

The CD14-/- C57BL/6 thymic cortex contained equivalent numbers of apoptotic cells as the wild type

(Figure 3.2c)

The importance of CD14 in the clearance of apoptotic cells was next examined in a different mouse strain. The C57BL/6 mouse differs from Balb/c in its susceptibility to leishmania, and it is used in mouse models of autoimmune diseases like EAE (experimental autoimmune encephalitis). This was an important experiment to do because the persistence of apoptotic cells in the PSR knockout animals depended on the particular strain of mouse. Isogeneic C57BL/6 mice did not have a clearance defect whereas the mixed 129/C57BL/6 had a persistence of apoptotic cells (Böse, 2004; Li, 2003).

The number of apoptotic cells in the thymi from six CD14-/- mice on the C57BL/6 background was compared to wild type. In contrast to the results from the BALB/c strain, there was no significant difference between the numbers of TUNEL positive cells in the cortex or medulla of the C57BL/6 CD14-/- mice compared to wild type controls (ns $p > 0.1$).

This may indicate a strain difference in the use of CD14 for the clearance of apoptotic cells. C57BL/6 may not rely on CD14 for clearance to the same degree as the BALB/c strain. It will be interesting to see whether a CD14-defect is exposed in the C57BL/6 strain after a dexamethasone challenge and whether the C57BL/6 macrophages have a clearance defect *in vitro*. There is also a difficulty comparing the BALB/c and C57BL/6 animals directly because both were housed in different conditions. The C57BL/6 mice were obtained from Jackson laboratories and were certified as being specific pathogen free and were kept in IVC cages whereas the BALB/c lived in conventional housing.

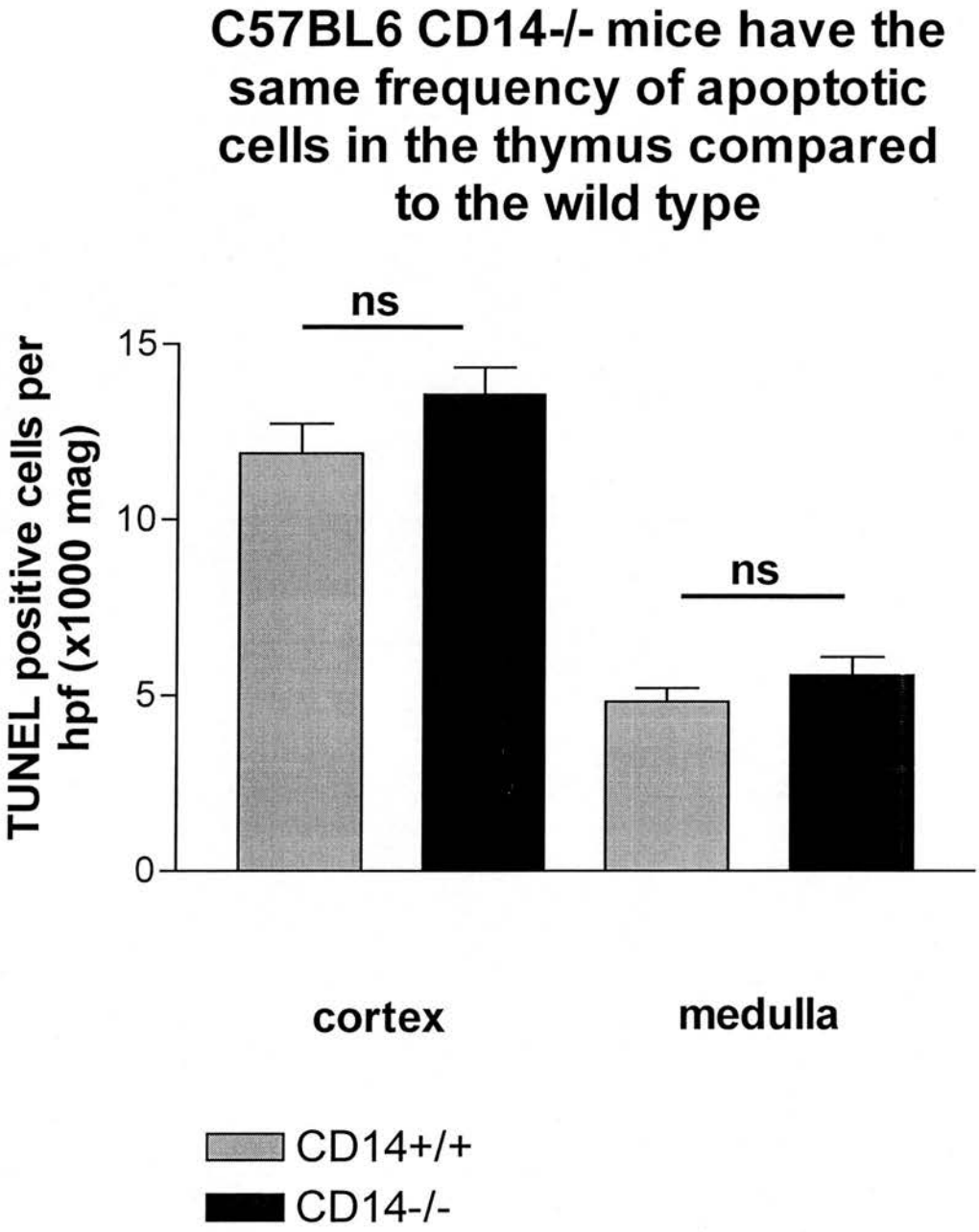


Figure 3.2c

The number TUNEL-positive cells in the medulla and cortex of thymus from CD14-/- (black, n=6) mice compared to wild type (grey, n=6).There was no significant difference between the number of apoptotic cells in the thymus of the CD14-/- and the wild type mice.

CD14-low monocytes do not up-regulate CD14 as they mature into macrophages *in vitro*

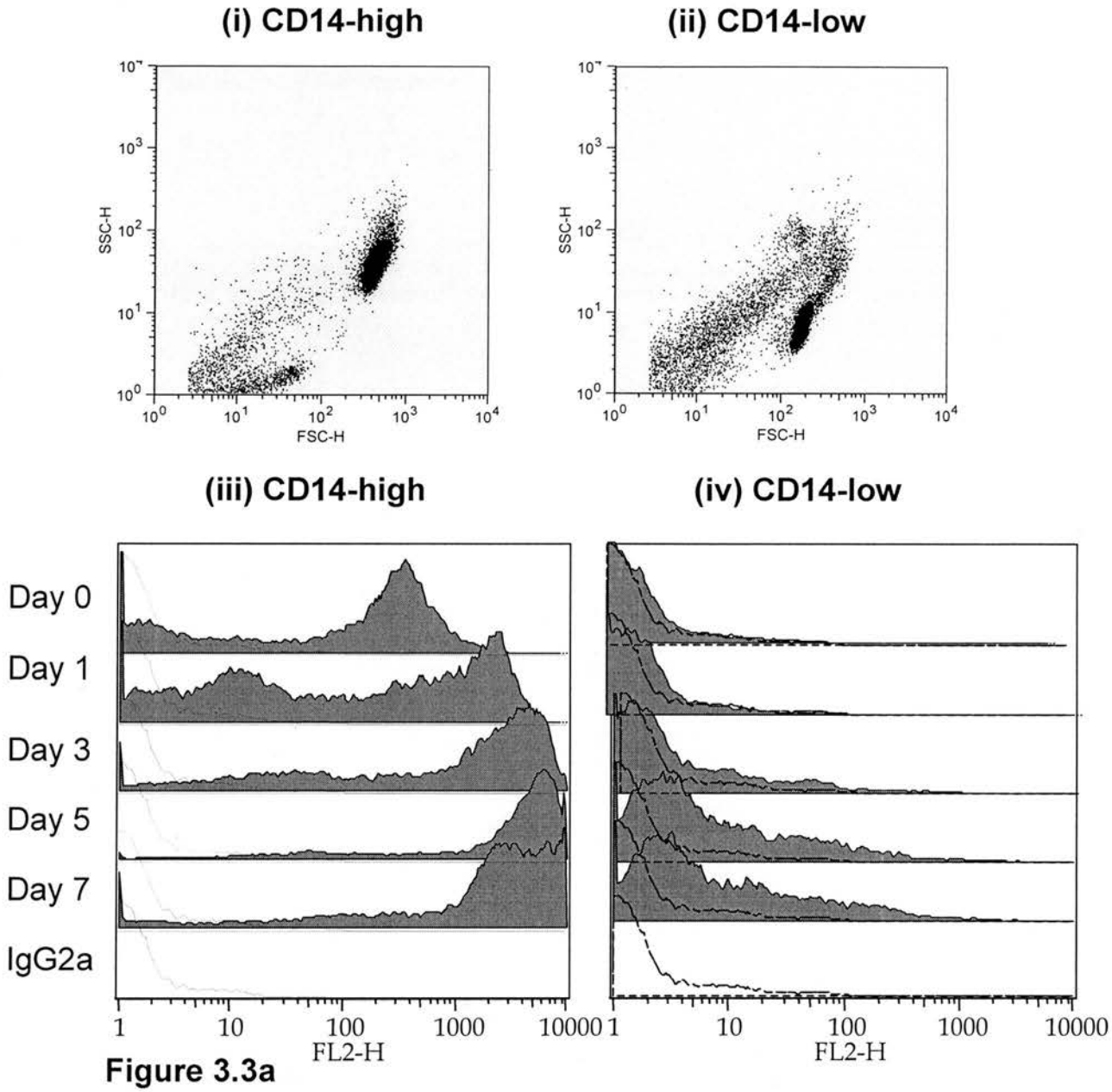


Figure 3.3a

Flow cytometry of CD14 expression on CD14-high and CD14-low monocytes as they matured into macrophages. Forward versus side scatter profile of CD14-high (i) versus CD14-low (ii) monocytes. Both monocytes were of equivalent size. Log-fluorescence intensity of PE-labelled CD14 expression on developing monocytes compared to isotype control (dashed line), in CD14-high (iii) compared to CD14-low (iv) monocytes.

3.3 $CD14^{low}$ monocytes from human peripheral blood mature into $CD14^{low}$ macrophages that have a defect in interactions with apoptotic cells

Next, the CD14 observations were extended to human macrophages. A population of human monocytes with low levels of CD14 that also express CD16 have been well characterised in the literature. However, the ability of these $CD14^{low}$ $CD16^{+}$monocyte-derived macrophages to phagocytose apoptotic cells had not been examined previously (Ziegler-Heitbrock, 1996). $CD14^{low}$ monocyte-derived macrophages were isolated from peripheral blood monocytes and their ability to bind to apoptotic cells was examined.

Both the $CD14^{high}$ and the $CD14^{low}$ human monocytes matured into macrophages that expressed CD68

(Figures 3.3a and 3.3b)

Monocytes were isolated from peripheral blood over a density gradient and then CD14 ^{high} monocytes were separated from CD14 ^{low} monocytes using magnetic bead separation. The $CD14^{high}$ and the $CD14^{low}$ fractions were seeded into culture dishes (for FACS) or onto glass slides (for immunohistochemistry) for one hour before the non-adherent cells were washed away.

After separation from peripheral blood, the $CD14^{high}$ and $CD14^{low}$ monocytes were analysed by FACS. Both the $CD14^{high}$ and the $CD14^{low}$ monocytes were of similar size and granularity as assessed by their forward and side scatter profiles on FACS (figure 3.3a). However, previous publications had shown that the $CD14^{low}$ monocytes were smaller than the $CD14^{high}$ monocytes (Ziegler-Heitbrock, 199).

The expression of surface CD14 on $CD14^{high}$ and the $CD14^{low}$ monocytes was analysed as they matured into macrophages *in vitro*. Figure 3.3a shows the log-fluorescence intensity of PE-labelled CD14 staining of monocytes as they matured compared to IgG2a isotype control. The $CD14^{high}$ monocytes maintained high levels of CD14 expression as they matured into macrophages. After one week in culture,

Macrophages derived from both CD14-high and CD14-low human monocytes expressed CD68

(i) CD14-high

(ii) CD14-low

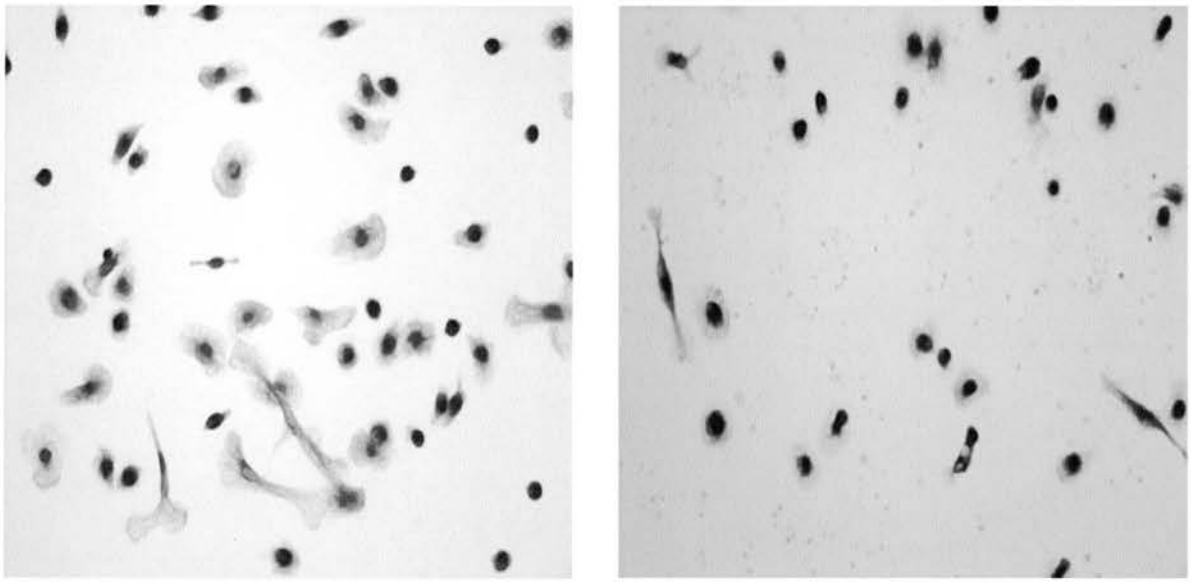


Figure 3.3b
Immunohistochemistry of 7-day-old human macrophages derived from CD14-high and CD14-low monocytes . Cells positive for the macrophage scavenger receptor CD68 were stained brown and nuclei were counter-stained blue with haematoxylin.

only a minority of the $CD14^{low}$ macrophages expressed CD14. This suggested that $CD14^{low}$ monocytes maintained their phenotype as they matured into macrophages.

Both the $CD14^{high}$ and the $CD14^{low}$ monocytes were able to adhere to glass slides and both stained equally well with the macrophage marker CD68 (figure 3.4b). Using this method of isolation, less than 5% of all the monocytes were $CD14^{low}$; this figure was in-line with published data (Ziegler-Heitbrock, 1996). The small number of $CD14^{low}$ macrophages obtained by this method was adequate to perform reproducible interaction assays. However a higher yield might have been obtained by using FACS sorting of immunolabelled cells.

$CD14^{low}$ human macrophages interact poorly with apoptotic cells compared to $CD14^{high}$ macrophages

(Figure 3.3c)

The phagocytic function of macrophages that had been matured from $CD14^{low}$ monocytes was compared to that of $CD14^{high}$ macrophages. Although $CD14^{low}$ macrophages appeared to have a similar phenotype to $CD14^{high}$ macrophages they bound significantly fewer apoptotic cells (**$p<0.001$) (figure 3.3c). Low levels of CD14 impaired macrophage phagocytosis of apoptotic cells. The result from human $CD14^{low}$ macrophages was in agreement with the findings of the Balb/c CD14 -/- mouse macrophages.

Figure 3.3c shows the average result of two macrophage and apoptotic cell interaction experiments. 27.67% of the macrophages that had matured from $CD14^{high}$ monocytes interacted with apoptotic cells. Significantly fewer of the $CD14^{low}$ macrophages bound apoptotic cells (12.98% **$p<0.001$). This preliminery data would be in line with previous data showing a possible role for CD14 in macrophage binding to apoptotic cells. (Devitt, 1998).

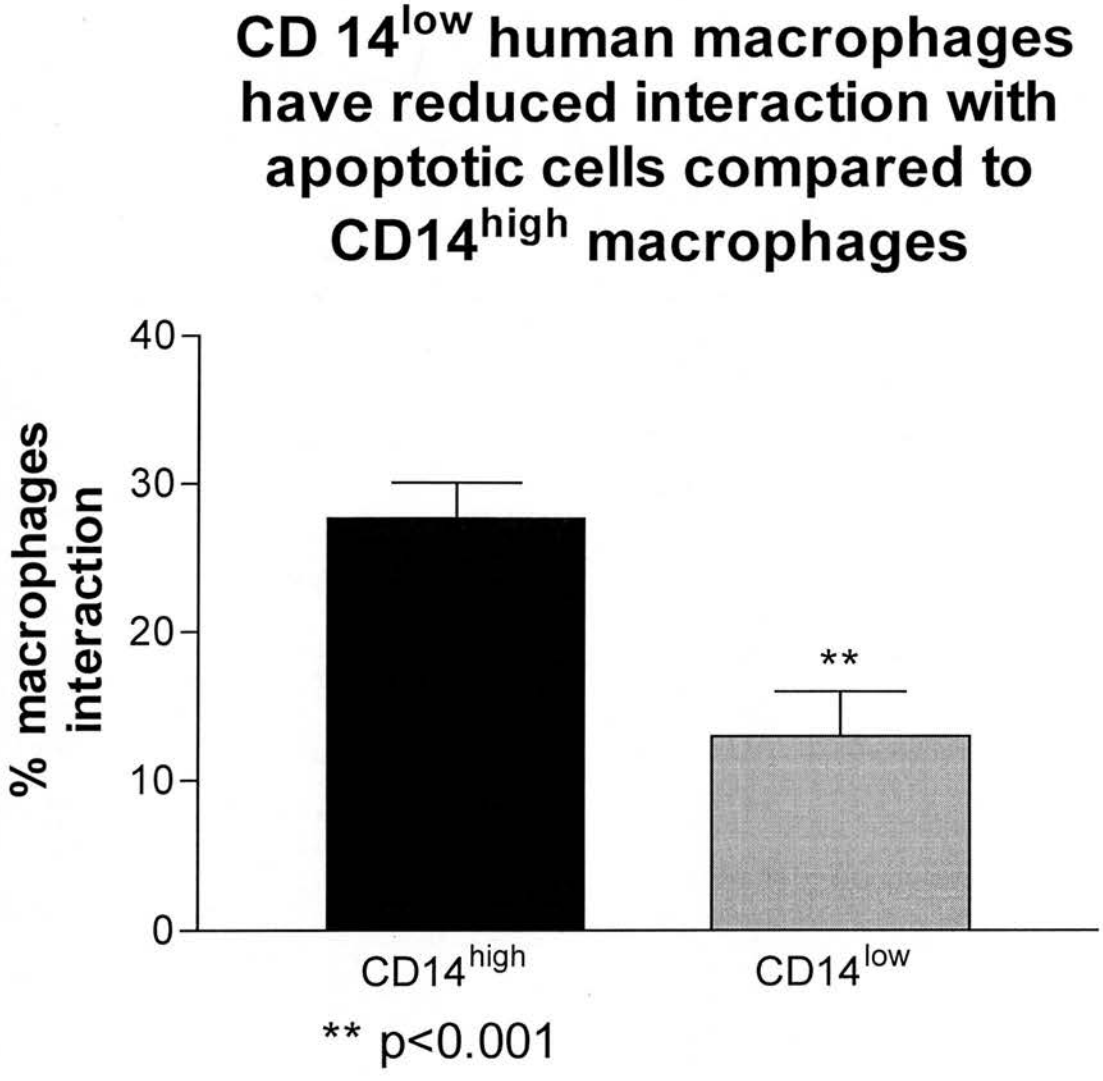


Figure 3.3c

Phagocytosis of apoptotic Burkitt's lymphoma cells (Mutu) by macrophages matured from CD14-high (black) and CD14-low (grey) monocytes. Significantly fewer CD14-low macrophages phagocytosed apoptotic Mutu compared to CD14-high macrophages (**p< 0.001, student's t-test) Mean data + SEM from four experiments was plotted.

Chapter 3 summary

- The CD14-/- BALB/c mouse has more "free" apoptotic cells in the thymic cortex than the wild type *in vivo*.
- Quantitative analysis of apoptotic cells by TUNEL labelling shows that the BALB/c CD14-/- mouse has increased numbers of apoptotic cells in the thymic cortex but the C57BL/6 does not.
- *In vitro* interaction assays showed that BALB/c CD14-/- macrophages had a partial defect in the clearance of apoptotic BL-cells
- Approximately 5% of human monocytes isolated from peripheral blood were $CD14^{low}$ and, like murine CD14 -/- BMDM, the $CD14^{low}$ human macrophages also had a defect in the phagocytosis of apoptotic cells

The results of this chapter confirmed published work indicating that CD14-/- macrophages on the Balb/c background had a clearance defect leading to an accumulation of apoptotic cells seen both *in vivo* and *in vitro*. Interaction assays confirmed that Balb/c CD14-/- macrophages had a defect in the phagocytosis of apoptotic cells. However, this defect was not observed in the C57/BL6 mouse. This may be due to the M1-like macrophages in the C57BL/6 strain or due to differences in the housing of the mice.

The first step in the clearance of apoptotic cells involves a macrophage "sensing" and then "moving towards" the dying cell, before finally phagocytosing it. Balb/c CD14-/- macrophage-phagocytosis of apoptotic cells was reduced, but not completely blocked. The CD14-/- on the Balb/c background had many "free" apoptotic cells that were not associated with macrophages. Therefore, it was hypothesised that, in addition to tethering defect, CD14-/- macrophages may also have a fault in the come-get-me stage of apoptotic-cell clearance. The next chapter will describe the role of CD14 in the chemotaxis of macrophages towards apoptotic cells.

CHAPTER 4

Macrophage chemotaxis to apoptotic cells

Introduction

The previous chapter showed that the Balb/c CD14-/- mouse had increased numbers of apoptotic cells in the thymus *in vivo*, which may have been due in part, to a defect in the tethering of apoptotic cells to macrophages. The large number of "free" apoptotic cells, those not associated with macrophages in the CD14-/- suggested there might also be a defect in these animals' "sensing" and "moving" towards apoptotic cells (Devitt, 2004). Experiments presented in this chapter have investigated the role of macrophage chemotaxis to apoptotic cells that might account for the accumulation of cell bodies seen in the CD14-/-.

While much has been learned in recent years of the mechanisms by which apoptotic cells are recognised and engulfed by macrophages, little is known of the processes underlying the 'sensing' of apoptotic cells by phagocytes. Recent studies have highlighted the importance of chemoattractant molecules that are released during normal cell death and of how macrophages respond to these come-get-me signals, in readiness to engulf the apoptotic corpse (Kao, 1994; Audran, 1996; Horino, 1998; Segundo, 1999; Cocca et al., 2002 and Lauber et al., 2003). Apoptotic-cell clearance might be delayed by faulty macrophage chemotaxis resulting in unwanted inflammation and a predisposition to autoimmune diseases like systemic lupus erythematosus, (Herrmann, 1998). An alternative view is that the persistence of apoptotic cells may prolong the anti-inflammatory responses that accompany their engulfment by macrophages (Devitt, 2004; Gregory, 2004).

Chemotaxis is the directional movement of a cell up a chemokine gradient and is important in a number of biological processes including leukocyte recruitment to the sites of inflammation, and wound healing (Zlotnik, 2000). This chapter examines the migration of macrophages to chemoattractant molecules released from apoptotic Burkitt's lymphoma (BL) cells. Migration and phagocytosis involve common membrane and cell-shape changes, and both these processes are regulated by shared signalling pathways that involve integrins and small G proteins (Hogg, 2001). Apoptotic-cell engulfment and cell migration in the nematode worm (*Caenorhabditis elegans*) has been shown to be governed by the same highly-conserved genes (Wu, 1998). Given the mechanistic similarities between phagocytosis and chemotaxis, it was reasoned that macrophage receptors involved in

the recognition and engulfment of apoptotic cells might also function in the chemotactic response of macrophages to such cells.

This chapter focuses on two receptors that have important roles in the clearance of apoptotic cells: the lipolysaccharide receptor, CD14 and the class B scavenger receptor, CD36. The work published by Lauber et al, (whilst the present work was in progress) demonstrated that the lipid, lysophosphatidylcholine (LPC) was a chemoattractant released by apoptotic cells (Lauber, 2003). It is significant that both CD14 and CD36 can bind lipids and so may be able to "sense" chemotactic molecules (like LPC) released by apoptotic cells.

Using cells established from Burkitt's lymphoma (BL) as a source of apoptotic cells, the transmigration assay described in Chapter 2, was used to measure macrophage chemotaxis to apoptotic BL-cells. Finally, experiments to characterise the chemoattractive molecule released by dying Burkitt's lymphoma cells will be described.

4.1 Macrophages are actively phagocytosing apoptotic-cells in Burkitt's lymphoma

(Figure 4.1)

Burkitt's lymphoma (BL) is a small non-cleaved B cell tumour that contains many macrophages diffusely distributed amongst the "monotonous sea" of tumour cells. Sections prepared from human tumours and stained with H&E (figure 4.1(i)) showed many apoptotic cells with typical shrunken, pyknotic nuclei (marked by an asterisk). Apoptotic cells were more intensely stained blue compared to neighbouring live cells. Pale-staining macrophages stood out from the surrounding dense nuclei of the tumour cells that created the characteristic "starry sky" appearance of the tumour seen at low power. The cytoplasm of the macrophages was engorged with apoptotic bodies (marked with an arrow), indicating that macrophages had actively phagocytosed apoptotic cells *in situ*. The high numbers of macrophages distributed throughout the tumour was striking when the cells were labelled with anti-CD68, a macrophage marker (figure 4.1(ii)).

Immunohistochemistry of macrophages in human Burkitt's lymphoma

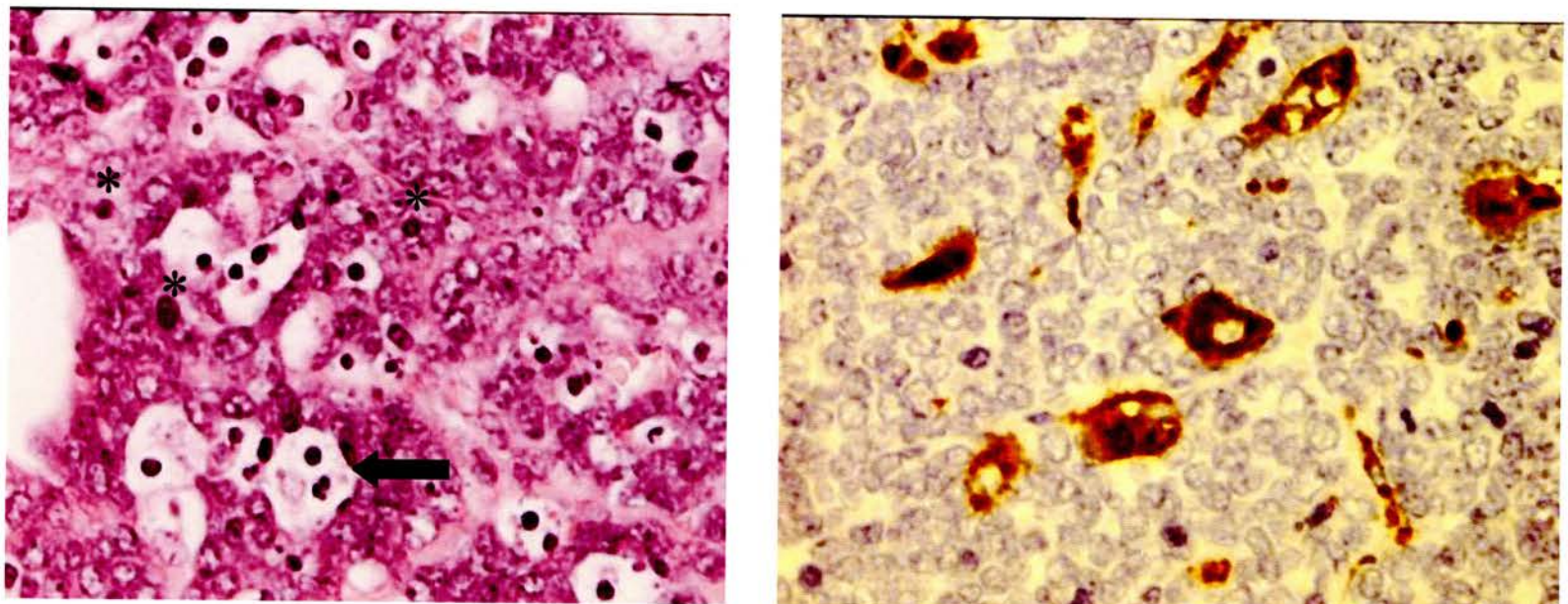


Figure 4.1

The typical "starry sky" appearance of tumour associated macrophages (TAM) in human Burkitt's lymphoma. Formalin fixed 5μm thick sections were stained with haematoxylin and eosin (i). Frequent apoptotic cells are seen with darkly-stained pyknotic nuclei labelled (*). TAM are less dense and lighter than the monotonous "sea" of tumour cells. Arrow points to a macrophage containing phagocytosed apoptotic bodies. (ii) Sections were immunolabelled with mouse anti-CD68 (clone PG-M1) followed by a biotinylated goat anti-mouse immunoglobulin secondary (clone). Positive cells were detected by a streptavidin-HRP and DAB colour reagent and sections were counter-stained with haematoxylin. CD68 positive macrophages are brown.

4.2 Macrophage chemotaxis to Mutu BL-cells

(Figure 4.2a)

Macrophage transmigration to Mutu BL-cells, containing varying amounts of apoptosis was measured using the transwell system. Mutu transfected with the anti-apoptotic gene *bcl-2* (Mutu $_{bcl\text{-}2}$) had the lowest numbers of apoptotic cells (15%), Mutu BL-cells (Mutu BL-cells) had intermediate levels of spontaneous apoptosis (23%), and 89% of UVB-treated Mutu BL-cells (Mutu $_{UVB}$) were apoptotic. 100ng/ml CCL5 in RPMI was used as a positive control and media alone as the negative control in all experiments.

Figure 4.2a is a representative example of 7 separate experiments and shows that week-old macrophages preferentially migrated to apoptotic rather than to live Mutu BL-cells. Macrophage chemotaxis to UVB-treated Mutu BL-cells was significantly higher than to Mutu BL-cells $_{bcl\text{-}2}$ (***$p<0.0001$, student's t-test). Transfection of Mutu BL-cells with *bcl-2* prevented Mutu BL-cell apoptosis and virtually abolished macrophage chemotaxis. Macrophage migration to untreated Mutu BL-cells depended on the number of (spontaneously) apoptotic cells present and was always higher than Mutu $_{bcl\text{-}2}$.and lower than Mutu $_{UVB}$.

Macrophage chemotaxis increases with apoptosis

(Figure 4.2b)

The number of apoptotic cells was carefully measured for every individual experiment because the percentage of apoptotic cells varied slightly each time. Apoptosis was measured by binding of FITC-labelled AxV and PI uptake by FACS and was confirmed by DAPI staining and fluorescence microscopy. This data collection allowed the rate of macrophage chemotaxis to be compared to the amount of apoptosis. Macrophage chemotaxis data from 7 experiments was collected together and was plotted against the percentage of Annexin V-positive Mutu BL-cells (figure 4.3b). The chemotactic index refers to the fold increase in the number of transmigrated macrophages above the negative (media) control. It can be seen that the chemotaxis index increased with the increase in the number of apoptotic cells.

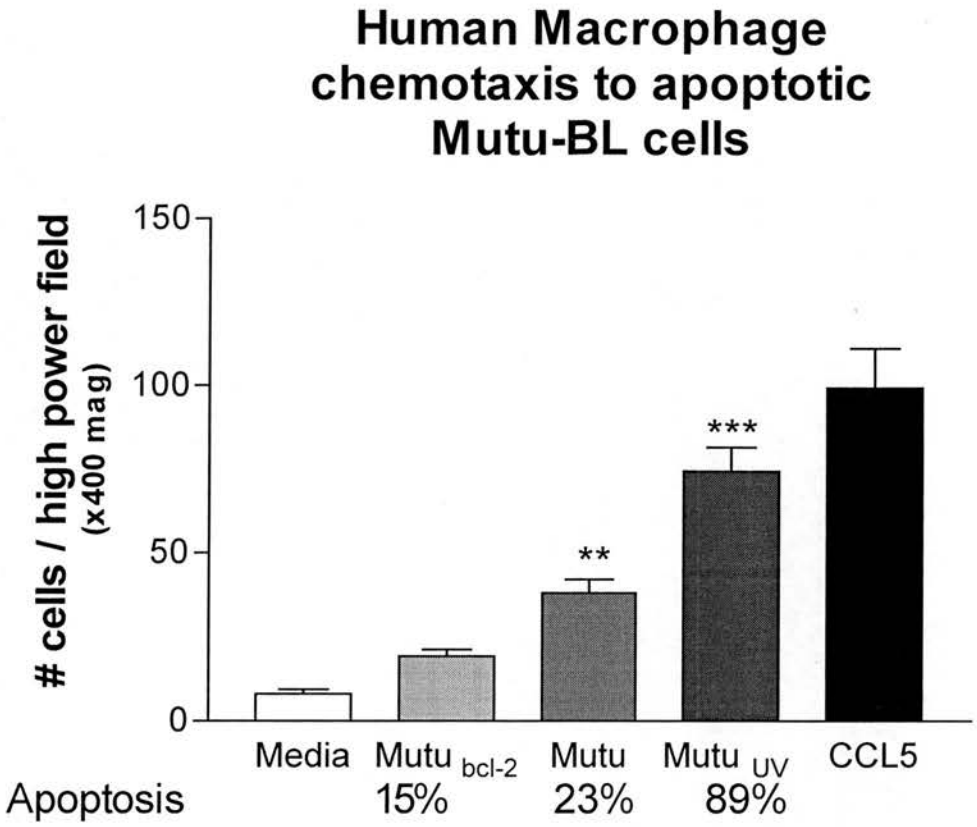


Figure 4.2a

Macrophages were allowed to transmigrate to media alone (negative control), or Mutu BL-cells with various levels of apoptosis, Mutu *bcl-2*, Mutu, UV-treated Mutu, or to 100ng/ml CCL5 (positive control) for 4 hours. The mean number (+SD) of macrophages transmigrating was counted for each condition. One representative experiment of 7 is shown. Macrophage chemotaxis to UV-treated Mutu BL-cells containing 89% apoptotic cells was significantly higher than to Mutu *bcl-2* containing 15% of apoptotic cells (***$p<0.0001$, student's t-test). % apoptosis is shown.

Macrophage chemotaxis to Mutu BL- cells increases with apoptosis

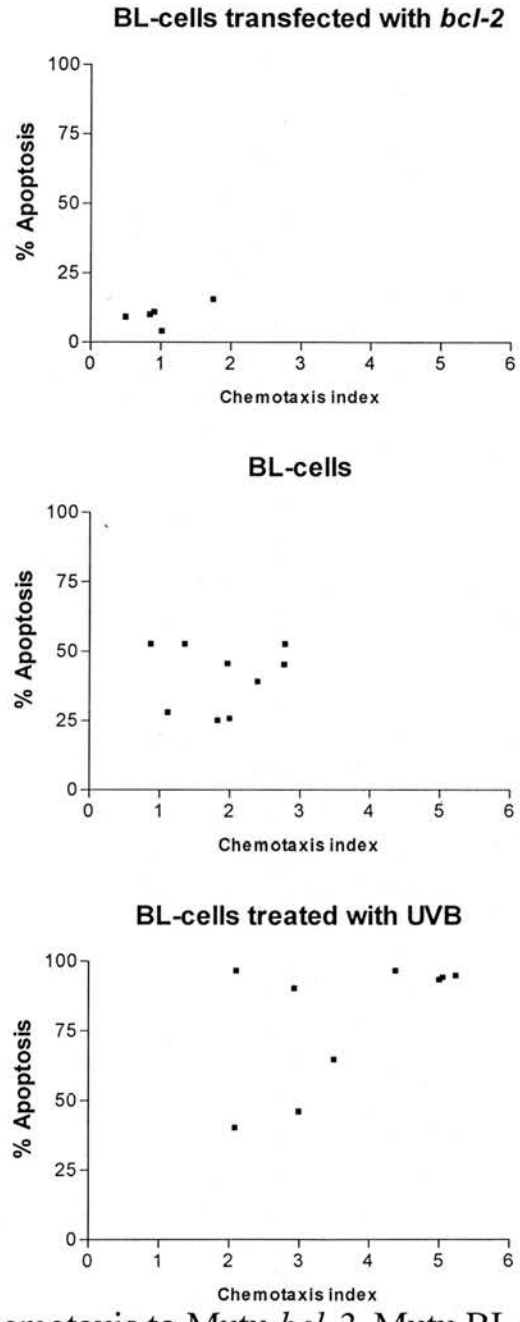


Figure 4.2b

Macrophage chemotaxis to Mutu *bcl-2,* Mutu BL cells, and UV-treated Mutu BL cells was compared to the number of apoptotic cells. Apoptosis was measured as the percentage of Mutu BL cell-binding FITC-labelled Annexin V analysed by flow cytometry. The chemotaxis index was the fold increase in the number of transmigrated macrophages compared to media alone (negative control).

This result showed that dying Mutu BL-cells released one or more chemoattractant molecules that attracted macrophages. The higher the number of AxV+ apoptotic cells, the greater was the number of macrophages that transmigrated.

4.3 Macrophage chemotaxis to apoptotic Mutu BL-cells in relation to the kinetics of apoptosis

(Figure 4.3)

A 24-hour time-course experiment was performed to determine at what time during programmed-cell-death apoptotic Mutu BL-cells released chemotactic molecules that recruited macrophages. Macrophages were allowed to migrate to Mutu BL-cells at different times after induction of apoptosis. Aliquots of 2x 10^6 cells/ml of Mutu BL-cells were treated with 100mJ/cm^2 UVB light at various times. Mutu BL-cells were washed, and placed in a 24 well plate. Macrophages were allowed to transmigrate for four hours and then the filters where counted as has been previously described. Figure 4.3 shows macrophage chemotaxis to apoptotic Mutu BL-cells at different time-points after the induction of apoptosis. Open circles show the baseline transmigration of macrophages to the media only (negative control) and the filled circles plot macrophage migration to UVB-treated Mutu BL-cells. There is an increase in macrophage transmigration as early as 4 hours after UVB-treatment. When macrophage migration is compared to the kinetics of Mutu BL-cell apoptosis it can be seen that macrophage migration to Mutu BL-cells occurred early after the induction of apoptosis, at about 4 hours, when the majority of cells were still in the viable zone (as shown by FACS). At this time-point, the majority of BL-cells had intact cell membranes and did not take up Propidium iodide. These results suggest that apoptotic cells actively released the chemotactic molecule, early on, whilst their membranes were still intact. Macrophage migration was sustained over the 24-hour period and did not tail off despite all Mutu BL-cells falling into the dead zone at these latter time points. This might suggest that apoptotic Mutu BL-cells first released an early burst of chemotactic molecules followed by a passive leak of chemotactic molecules at the later time points.

Kinetics of macrophage chemotaxis to apoptotic Mutu BL-cells

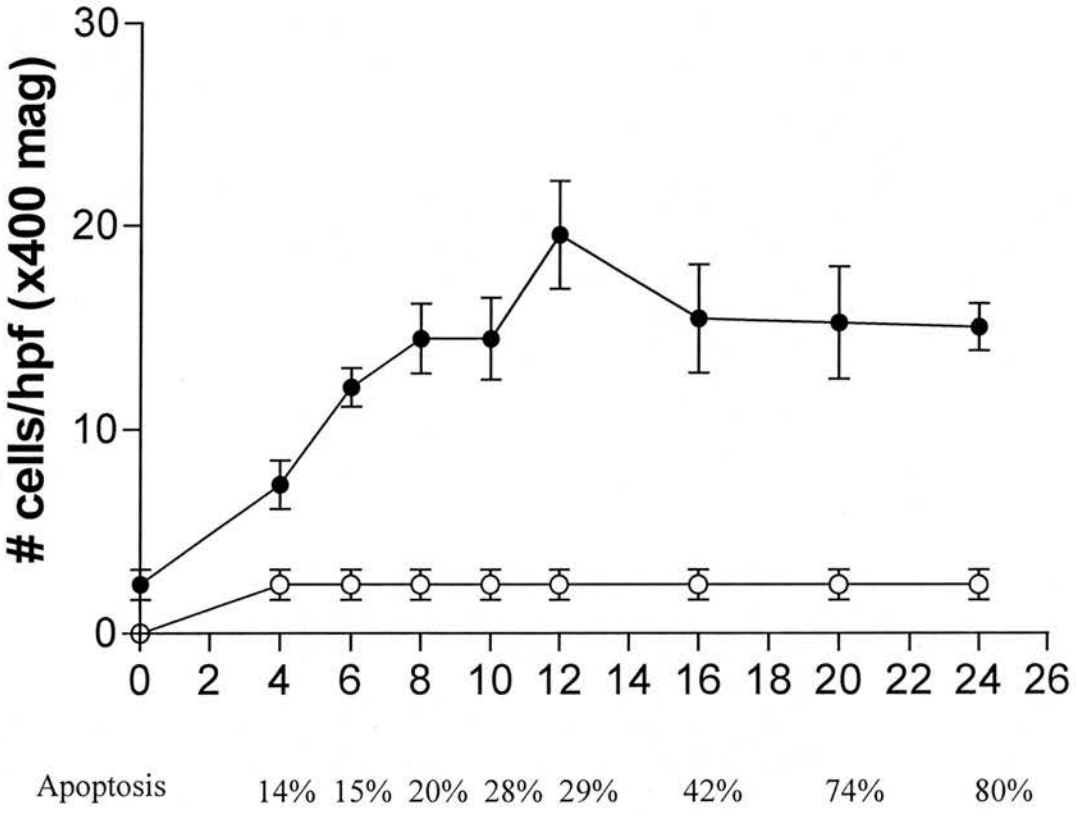


Figure 4.3
Mutu were treated with 100mJ/cm2 UVB to induce apoptosis and were harvested at various time points over 24 hours. Macrophages were allowed to migrate to to Mutu (filled circles) for 4 hours or to media alone (empty circles). The mean (+SD) of macrophages transmigrated of ten high-power fields at x400 magnification is shown. A representative result from two experiments is plotted. The percentage of apoptotic cells shown.

4.4 Macrophage chemotaxis to "leaky" or necrotic BL-cells

(Figure 4.4)

The next objective was to determine if macrophage transmigration was specific to apoptotic cells in particular, or to dying cells in general. The "danger hypothesis" stated that, in the absence of infection, endogenous cell products can cause inflammation and DC maturation (Gallucci, 1999). Spilled cell contents from dying cells may contain many danger signals, not least uric acid, an endogenous adjuvant in its own right (Shi, 2003). These spilled intracellular contents can be chemoattractive to leukocytes. Intracellular components like ATP and heat shock protein gp96 can induce chemotaxis (Honda, 2001; Binder, 2000). The previous experiments suggested that Mutu BL-cells released chemotactic molecules about 4 hours after the induction of apoptosis and this release continued when the cells became leaky Therefore, macrophage transmigration to leaky, necrotic cells was compared to apoptotic cells.

Mutu BL-cells were boiled for 20 minutes, and after this treatment, 100% of the cells were necrotic and trypan blue positive. Macrophages were allowed to migrate to Mutu BL-cells, apoptotic Mutu BL-cells and necrotic Mutu BL-cells. As shown previously, macrophages preferentially migrated to apoptotic Mutu BL-cells compared to live Mutu BL-cells (Figure 4.4). Macrophages migrated significantly more to the necrotic Mutu BL-cells, compared to apoptotic Mutu BL-cells that had intact cell membranes (***$p < 0.0001$, student's t-test). This suggested that, after an initial release, chemoattractant molecule continued to passively leak out of necrotic cells. This mechanism might maintain the concentration gradient allowing a sustained macrophage migration to cells that have begun to disintegrate. Indeed, *in vitro* studies show that macrophages phagocytose leaky apoptotic cells very efficiently (Turner, 2003).

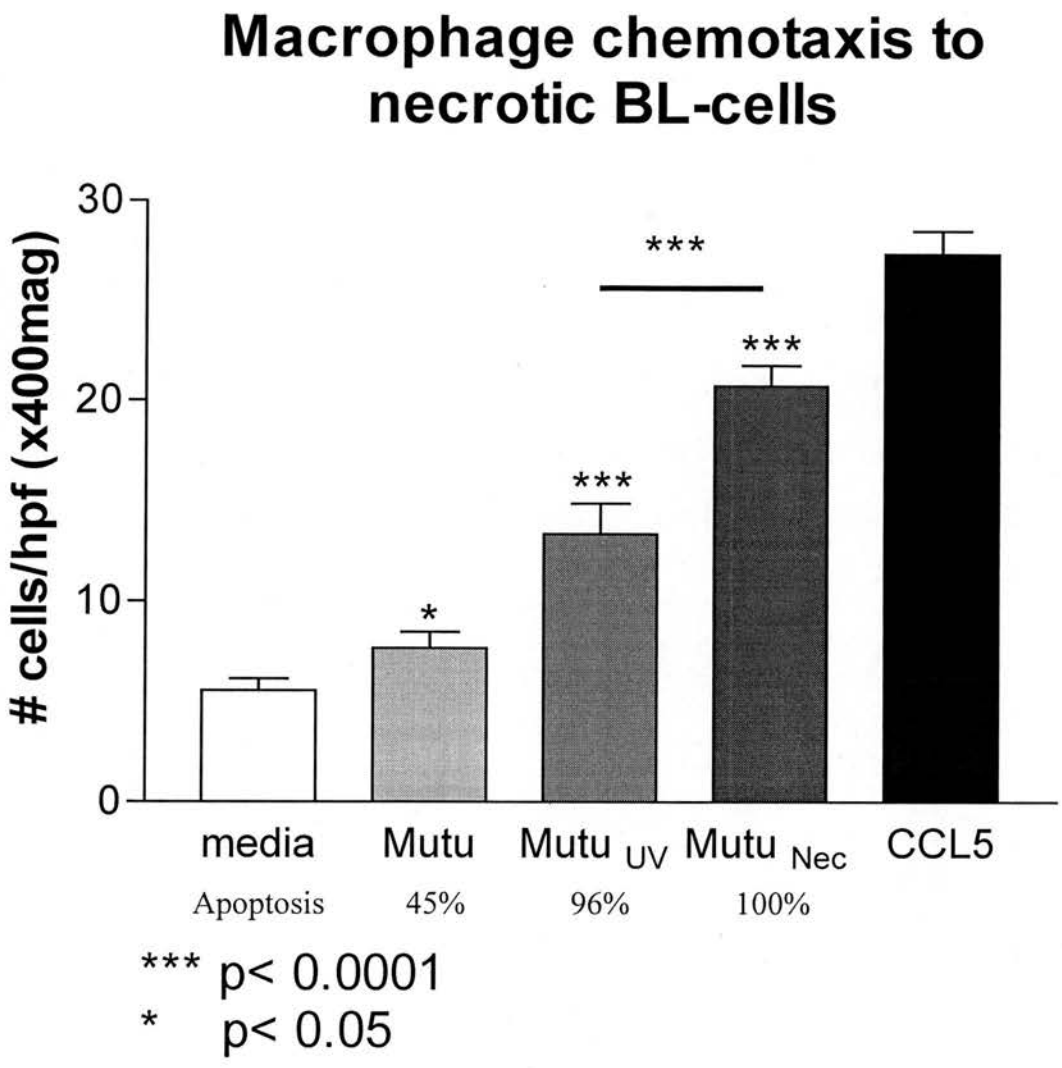


Figure 4.4

Macrophages migrated to Mutu BL-cells, UV-treated BL-cells, necrotic Mutu BL-cells, media only (negative control) and 100ng/ml CCL5 (positive control). The mean (+SEM) number of transmigrated macrophages for each condition is plotted. One representative experiment of two is shown. Macrophages preferentially migrated to apoptotic Mutu BL-cells compared to viable Mutu (***$p<0.0001$, student's t-test). There was a significant increase in the number of macrophages that migrated to necrotic Mutu compared to apoptotic (UV-treated) Mutu, (***$p<0.0001$, student's t-test). % number of apoptotic cells is shown for each treatment.

4.5 Chemotaxis of monocytes and neutrophils to apoptotic BL-cells

The previous sections dealt only with macrophage chemotaxis to apoptotic cells. Macrophages are commonly seen infiltrating Burkitt's lymphoma, but neutrophils are seen very rarely in the tumour (Wilkins, 2005). With this in mind, the transmigration of other leukocytes to apoptotic Burkitt's lymphoma was investigated. Examination of monocytes and neutrophils would show whether or not the receptors employed in chemotaxis to apoptotic cells were specific to macrophages.

Monocyte chemotaxis to apoptotic Mutu BL-cells

(Figure 4.5a)

Experiments were performed to see if primary monocytes could also transmigrate to apoptotic Mutu BL-cells. Primary human monocytes were isolated from human blood, separated on a Ficoll gradient and allowed to adhere to plastic for 1 hour. Non-adherent cells were washed off and adherent monocytes were used immediately in a transmigration assay. Figure 4.5a is a representative example of three experiments showing transmigration of monocytes to Mutu BL-cells. 100ng/ml of CCL5 was used as a positive control and media alone served as a negative control. Monocytes, like macrophages, preferentially migrated to apoptotic Mutu BL-cells, rather than Mutu BL-cells that had been transfected with *bcl-2* (***$p<0.0001$, student's t-test).

Overall, monocytes transmigrated less well to apoptotic Mutu BL-cells compared to macrophages. Monocytes may express lower levels of chemokine receptors than macrophages, or perhaps cells were damaged whilst being lifted out of plates before use in the transmigration assay. An alternative strategy would have been to use antibody-coated magnetic beads to purify monocytes and store them in teflon-coated tubes until use. Lauber et al, had already shown chemotaxis of primary monocytes to apoptotic cell supernatants using this approach and also observed that primary monocyte transmigration to apoptotic cell-lines was less efficient than that of macrophages (S.Wesselborg, personal communication, (Lauber, 2003).

Monocyte, MM6 and neutrophil chemotaxis to apoptotic BL-cells

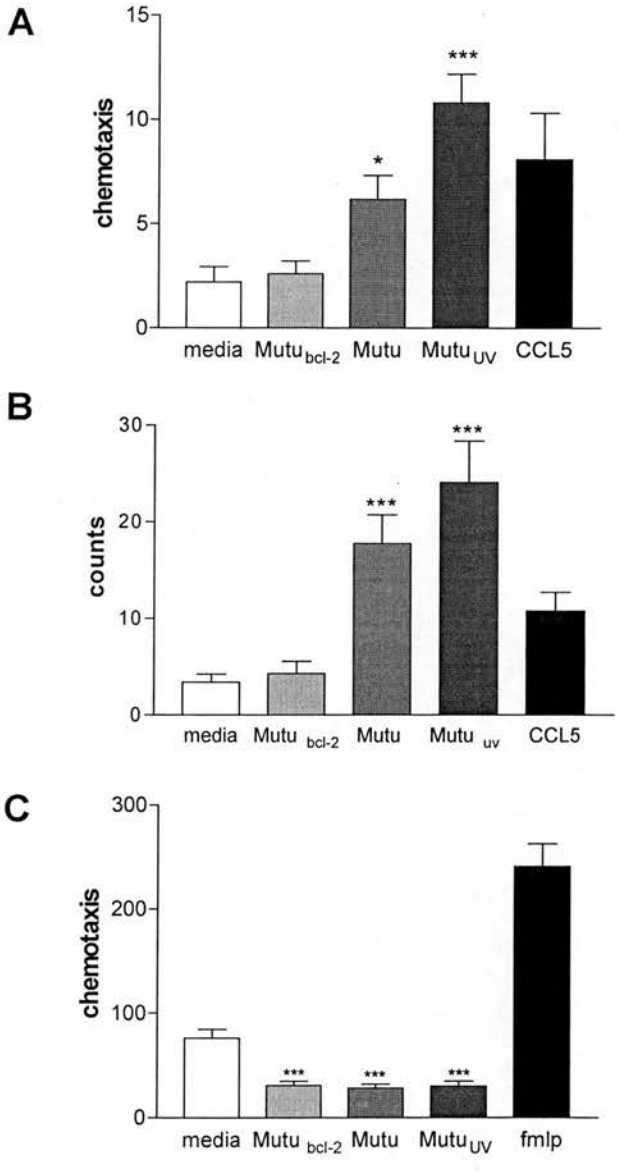


Figure 4.5

Monocyte (A), MM6 (B) and neutrophil (C) chemotaxis to Mutu *bcl-2,* Mutu and UV-treated Mutu BL-cells. Apoptosis was induced by UV-light. Chemotaxis in monocytes and neutrophils was measured as the mean number of transmigrated cells counted per high power field. Chemotaxis in MM6 was measured by counting the number of transmigrated cells by FACS. The mean number (+SEM)of migrated cells was plotted for each condition. One representative result from at least three separate experiments is shown. There was no neutrophil chemotaxis to apoptotic Mutu. students t-test ***$p<0.0001$, **$p< 0.005$, * $p< 0.02$.

A monocyte cell line, THP-1 was used in lieu of primary monocytes in many of the experiments to overcome the problem of the low mobility of primary monocytes (Lauber, 2003). Likewise, in the next experiment, the transmigration of another monocytic cell-line MonoMac 6 (MM6) was measured.

Mono Mac 6 (MM6) chemotaxis to apoptotic Mutu BL-cells

(Figure 4.5b)

MM6 is a human monocyte cell line established from a myeloid leukaemia (Medzhitov, 1997; Ziegler-Heitbrock, 1988). MM6 are non-adherent in culture and can be matured into macrophages using PMA and vitamin D_3. MM6 were used to examine monocyte chemotaxis to apoptotic cells.

MM6 were placed in the top chamber of a transwell experiment and allowed to transmigrate towards apoptotic Mutu BL-cells in the lower chamber. MM6 were non-adherent and passed through the filter into the lower chamber and so were counted by FACS. Figure 4.5b shows the results from three experiments. Chemotaxis index refers to the fold increase in the number of MM6 that migrated to Mutu BL-cells compared to the media (negative control). MM6 preferentially moved towards apoptotic Mutu BL-cells compared to Mutu BL-cells $_{bcl\text{-}2}$. The numbers of MM6 that transmigrated to Mutu BL-cells and UVB-treated Mutu BL-cells was significantly higher than to Mutu $_{bcl\text{-}2}$ (***$p<0.0001$, student's t-test). Increased numbers of MM6 cells moved towards apoptotic cells BL-cells, similar to the pattern that had been observed in macrophages and primary monocytes.

Polymorphonuclear leukocyte chemotaxis to apoptotic Mutu BL-cells

(Figure 4.5c)

In stark contrast to monocytes and macrophages, neutrophils failed to migrate to Mutu BL-cells, despite migrating effectively towards 10^{-8}M fMLP (Figure 4.5c). At the end of the experiment, over 90% of the neutrophils were viable (as determined by tryphan blue exclusion) at the end of the short (1 hour) migration period. Cytospin preparations of neutrophils also showed no evidence of degranulation. Mutu BL-cells were not chemoattractive to neutrophils and appeared to be capable of actively preventing neutrophil transmigration, presumably through the release of soluble

inhibitory-factors. Failure of neutrophil chemotaxis to Mutu BL-cells appeared to be independent of apoptosis, since, Mutu $_{bcl\text{-}2}$, (with low levels of apoptosis) and Mutu $_{UVB}$, containing large numbers of apoptotic cells, both inhibited chemotaxis.

These *in vitro* findings reflected the number of neutrophils seen in Burkitt's tumours *in vivo*. The histological picture of Burkitt's lymphoma, shown in figure 4.1, shows a paucity of infiltrating polymorphonuclear cells. The observation that neutrophils failed to migrate to apoptotic Mutu BL-cells requires further work. One prediction might be that Mutu BL-cells failed to secrete CXCL chemokines. This chemokine group express the ELR (Glutamate-Leucine Arginine) tripeptide, a sequence that specifically attracts neutrophils. The role of two phagocyte receptors both expressed by macrophages, but not by neutrophils, in the chemotaxis to apoptotic BL-cells was investigated next.

4.6 The role of CD14 and CD36 in macrophage chemotaxis to apoptotic BL-cells in vitro

Having shown that apoptotic cells, but not neutrophils attracted macrophages the role of CD14 and CD36 was investigated. There are many mechanistic similarities between phagocytosis and chemotaxis and it is possible that macrophage receptors involved in the recognition and engulfment of apoptotic cells might also function in the chemotactic response to dying cells. Recently, the lipid lysophosphatidylcholine (LPC), released by apoptotic cells was shown to function as a chemoattractant for macrophages (Lauber et al. 2003). As CD14 can bind phospholipid its role in macrophage chemotaxis to apoptotic cells was investigated.

The lipid receptor, CD14 has an important role in macrophage phagocytosis of apoptotic cells *in vivo*. Chapter 3 described how Balb/c CD14-/- mice had increased numbers of "free" apoptotic cells in the thymus and how this might be explained because the CD14-/- macrophages had failed to migrate towards apoptotic cells. To determine whether the CD14 was important in "sensing" the presence of a dying cell, the role of macrophage CD14 in chemotaxis to apoptotic BL-cells was examined.

Macrophages that have transmigrated to apoptotic cells express high levels of CD14

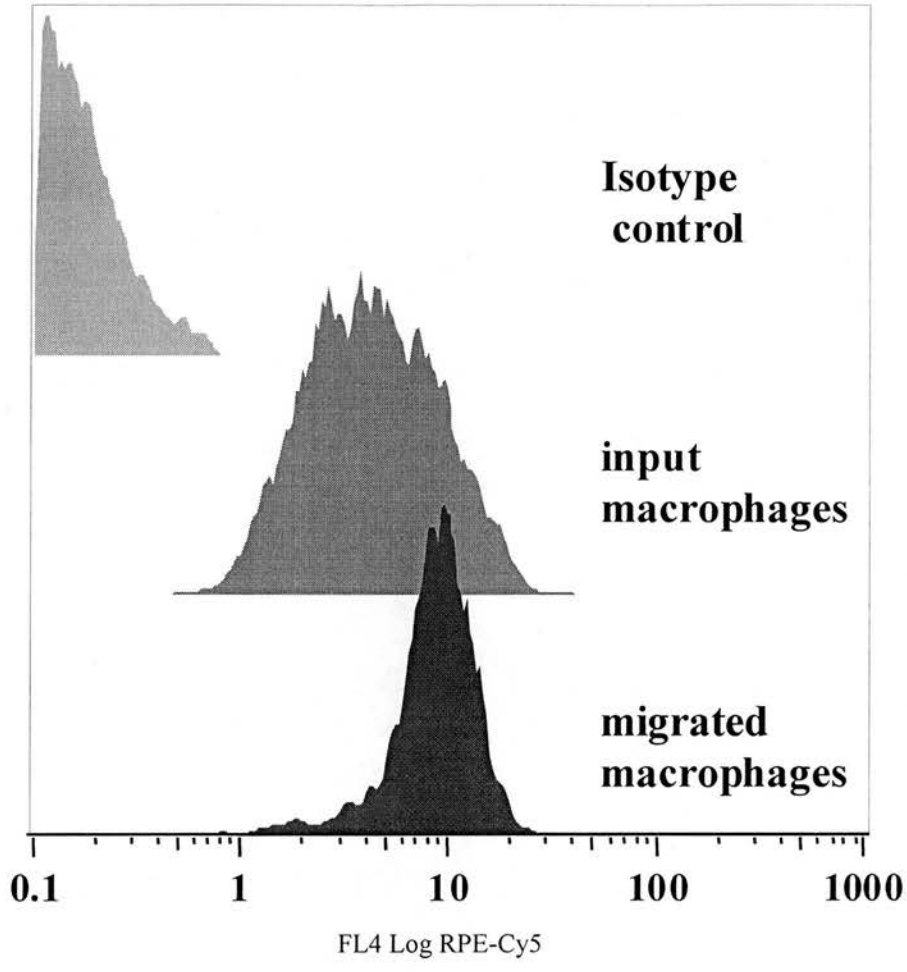


Figure 4.6a

Transmigrated macrophages expressed high levels of CD14. Macrophages were harvested from the underside of the transwell filter, after transmigration to apoptotic Mutu. Transmigrated macrophages were compared to input macrophages (pale blue). Macrophages were labelled with the anti-CD14 monoclonal antibody (TuK2). A mixture of input and transmigrated macrophages was labelled with the IgG2a isotype control antibody. Log fluorescence RPE-Cy5 intensity is plotted against relative cell number. One representative experiment of two is shown.

Human macrophages that have transmigrated to apoptotic BL-cells express high levels of CD14

(Figure 4.6a)

The level of CD14 expression was measured on transmigrating human macrophages as an initial indicator of a possible role for CD14 in chemoattraction of human macrophages. Mutu BL-cells were labelled with a fluorescent green marker at the start of the experiment so that they could easily be differentiated from macrophages during subsequent FACS analysis. The CD14 expression of macrophages that had transmigrated in response to apoptotic cells was compared to that of macrophages at the start of the experiment (input macrophages). After removing macrophages from the top chamber, macrophages that had transmigrated in response to apoptotic Mutu BL-cells were lifted off the underside of the filter by soaking in 5mM EDTA, spun down, collected and labelled with mouse anti-human-CD14 antibody. Fluorescent green, Mutu BL-cells were easily discriminated from macrophages by both fluorescence (log CM-green-FL1) and light scatter properties, and were gated out by FACS.

Figure 4.6a shows surface expression of CD14 on macrophages (log-CD14–Cy5-FL4 immunofluorescence). Macrophages that had transmigrated to apoptotic Mutu BL-cells expressed higher mean levels of CD14 than the input macrophages. It is possible that a $CD14^{high}$ population of macrophages had been specifically recruited to apoptotic Mutu BL-cells. Alternatively, the subset of macrophages that initially expressed low levels of CD14 at the beginning of the experiment ($CD14^{low}$), up-regulated CD14 during transmigration. It can be seen that CD14 was not globally up regulated on all macrophages, because the histogram for the transmigrated macrophages did not shift to the right. Therefore, it is most likely that it was the $CD14^{high}$ cells within the input population that had responded to the apoptotic cells. However this experiment did not demonstrate a role for CD14 in this chemotactic process.

CD14-/- and CD36-/- mouse bone marrow-derived macrophages (BMDM) have no defect in chemotaxis to apoptotic Mutu BL-cells

(Figure 4.6b)

If CD14 were important in the "sensing" and "moving towards" apoptotic cells, anti-CD14 antibodies would be expected to inhibit human macrophage chemotaxis. Preliminary studies using whole antibodies against human CD14 showed that anti-CD14 did prevent macrophage transmigration to apoptotic Mutu BL-cells whereas an isotype control antibody had no effect. However, Fab fragments would be required to test the specificity of the anti-CD14. It is possible that antibody complexes binding to macrophages may have caused a steric hindrance to movement. To avoid this difficulty macrophages from mice deficient in CD14 were used in transmigration assays.

The assay used for human cells was adapted in order to use mouse bone marrow-derived macrophages (BMDM). 10^{-8}M fMLP was used as positive control instead of human CCL5. In a similar way to human monocyte-derived macrophages, mouse BMDM migrated to apoptotic Mutu BL-cells. Mouse Balb/C BMDM migrated to UVB–treated Mutu BL-cells significantly more than to Mutu BL-cells that had been transfected with *bcl-2* (* $p<0.05$, student's t-test).

CD36 can bind lipids and like CD14, might also be a candidate for a macrophage chemotactic receptor. Since the CD36-/- mouse is on the C57BL/6 background, the transmigration assay was adapted once more to investigate C57BL/6 mouse. There was no strain difference between mice the chemotaxis to apoptotic cells using this assay. This species crossover showed that chemotaxis to apoptotic cells was a conserved phenomenon between the humans and mice. Therefore, CD14-/- macrophages could be used to test the hypothesis that CD14 had a role in macrophage chemotaxis to apoptotic cells.

Figure 4.6b shows that BALB/c mouse macrophages, that were deficient in the phagocytic receptor CD14, had no defect in chemotaxis to apoptotic Mutu BL-cells. Although important for the phagocytosis of apoptotic cells, this result indicated that CD14 was not necessary for macrophage chemotaxis to apoptotic cells. And likewise, CD36-/-have normal chemotaxis to apoptotic BL-cells.

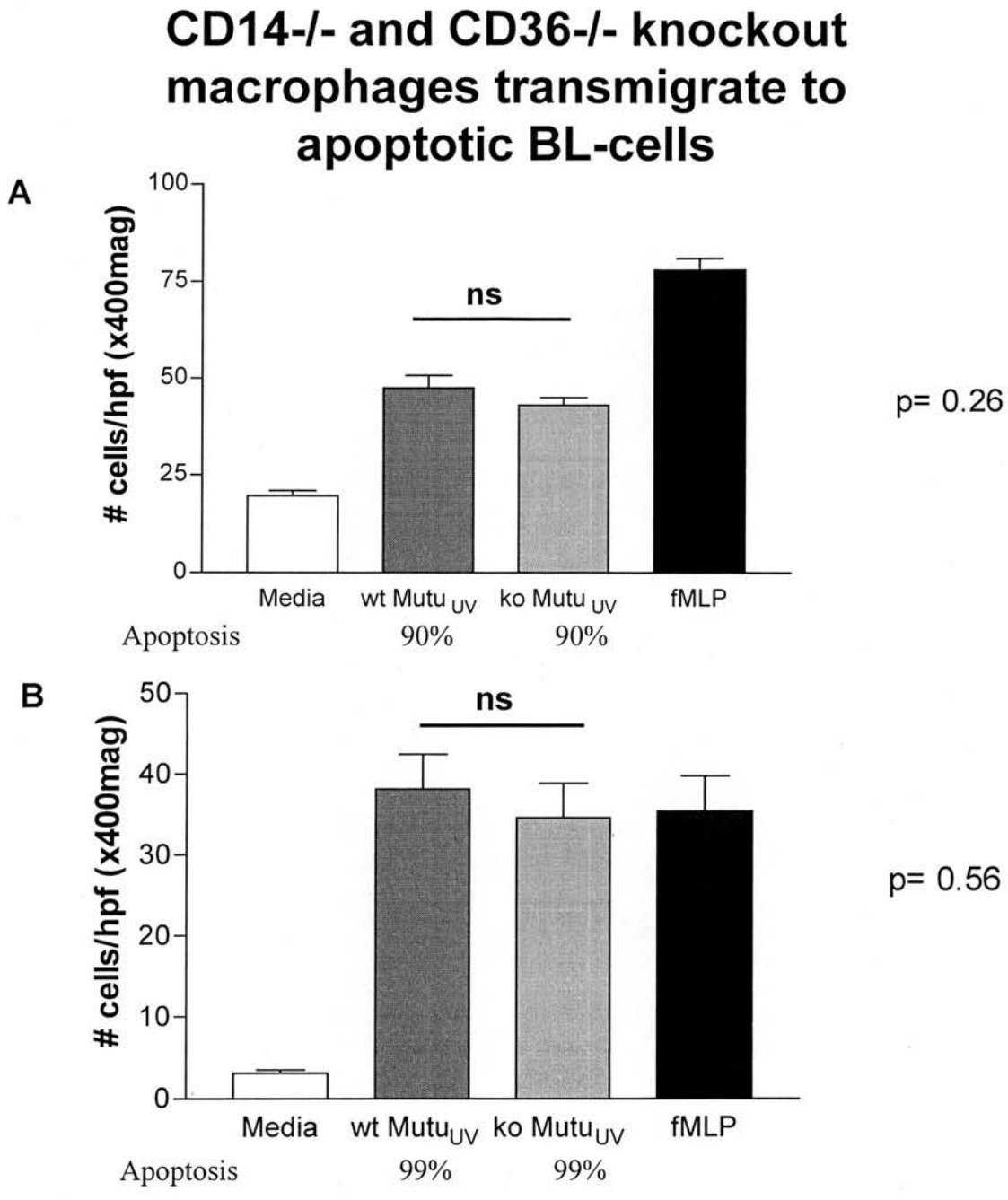


Figure 4.6b

BMDM from the CD14-/- (A) and the CD36-/- (B) and respective wild type controls (+/+) were allowed to transmigrate to Media alone (negative control), UV-induced apoptotic Mutu and 10-8M fMLP (positive control). Transmigration of both -/- and +/+ BMDM to fMLP is plotted. One representative experiment of four is shown and the mean (+SD) for each condition is plotted. There was no significant difference between CD14-/- and wild type macrophage transmigration (p=0.26). And there was no significant difference between CD36-/- and wild type macrophage (ns p=0.56, student's t-test). % number of apoptotic cells shown.

4.7 Macrophage chemotaxis to apoptotic BL-cells was not due to Epstein Barr Virus

(Figure 4.7)

The last section of this chapter will seek to characterise the chemotactic molecules that are released by apoptotic Mutu BL-cells. Although all experiments were performed in sterile conditions, each Mutu BL-cells carried a potential danger signal because of the presence of a latent infection with the Epstein-Barr virus (EBV). EBV can have profound immunomodulatory effects on cells both *in vitro* and *in vivo* that might additionally affect the secretion of chemotactic molecules. For example, EBV positive Hodgkin's lymphomas have increased secretion of chemokines (CCL5 (RANTES) and CXCL10 (IP-10)), whereas mononuclear cells from EBV-infected individuals are unable to produce CCL3 (MIP1-α) even after stimulation with LPS (Teruya-Feldstein, 2000 and Jabs, 2002).

In order to assess whether EBV infection influenced macrophage chemotaxis to apoptotic cells, an EBV negative Burkitt's lymphoma cell line, BL2 was compared to Mutu BL-cells. Figure 4.7 shows a typical result of macrophage chemotaxis to Mutu $_{bcl\text{-}2}$ Mutu BL-cells and apoptotic Mutu BL-cells compared to the EBV negative cells. Additionally, BL2 were transfected with the same *bcl-2* construct that had been transfected into Mutu BL-cells (by Carol-Anne Ogden). Like the Mutu $_{bcl\text{-}2}$, BL2 $_{bcl\text{-}2}$ also had low levels of spontaneous apoptosis. Figure 4.7(ii) is a representative example of five experiments and shows that macrophages also transmigrated to the EBV-negative cell line, BL2. Macrophages preferentially migrated to apoptotic BL2 compared to BL2 $_{bcl\text{-}2}$, similar to Mutu BL-cells.

These results demonstrated that macrophages migrated preferentially to apoptotic cells from two different BL-cell lines. This means that EBV is not needed for macrophage chemotaxis in this model but it remains possible that EBV stimulates the production of chemotactic molecules in the EBV positive Mutu BL-cells.

EBV infection did not affect macrophage transmigration to apoptotic BL-cells

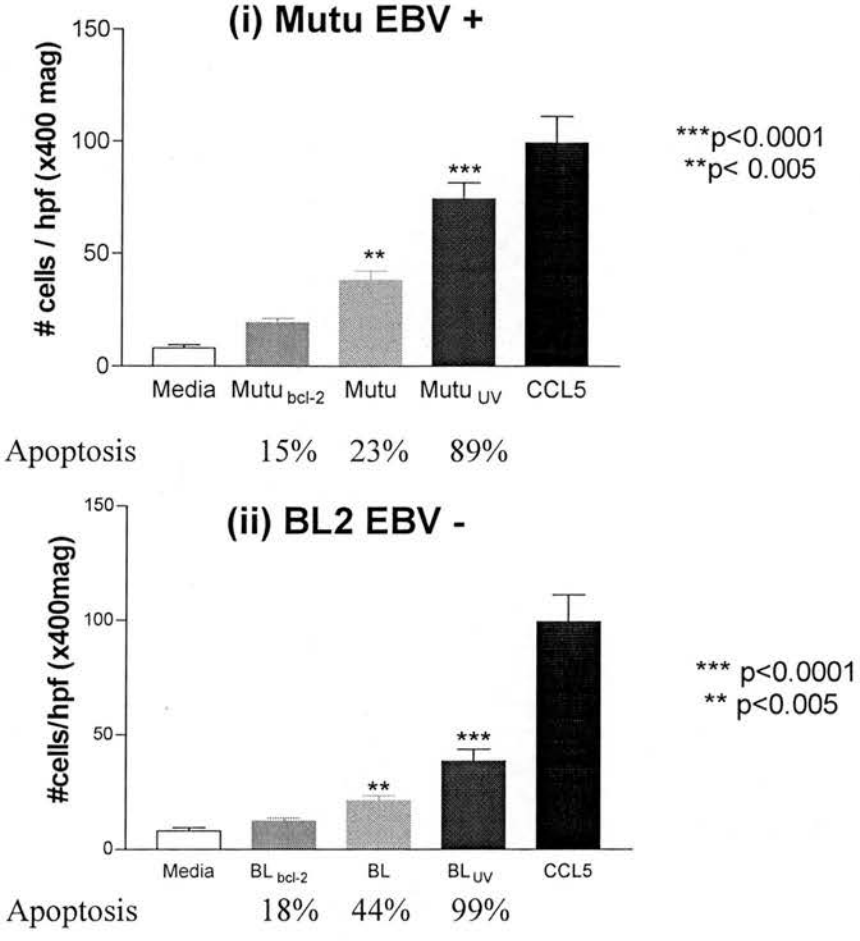


Figure 4.7

Apoptosis of cell lines was induced by UV-light. Macrophages were allowed to transmigrate to *bcl-2* transfected, untransfected live, UV-treated BL-cells, media only (negative control) and 100ng/ml CCL5 (positive control). The mean number (+SEM) of transmigrated cells counted per high power field, for each condition was plotted.One representative result from at least three separate experiments is shown. Macrophage chemotaxis to apoptotic, UV-treated BL-cells was significantly higher than to untreated BL-cells. Macrophage chemotaxis to apoptotic BL occurred in both EBV positive and EBV negative cells. % of apoptotic cells is shown.

4.8 The method of induction of apoptosis does not affect macrophage chemotaxis to apoptotic BL-cells

(Figure 4.8)

It could be argued that the mode of induction of apoptosis gave a shock to the cell that itself affected the release of chemotactic molecules. For this reason, spontaneously apoptotic Mutu BL-cells were compared to cells induced by other apoptotic stimuli for their ability to attract macrophages. Macrophages were able to transmigrate to spontaneously apoptotic cells, suggesting that chemotaxis was to common products released during programmed cell death and was not dependent on the stimulus that induced cell apoptosis. In order to further assess whether the method of induction of apoptosis affected the rate of macrophage chemotaxis to apoptotic cells, different chemical methods of inducing apoptosis were compared to treatment with UVB-light (figure 4.8). UVB-light treatment was compared to chemical induction of apoptosis using ionomycin, actinomycin D and staurosporine. Actinomycin D is a drug that can bind to DNA and block the movement of RNA polymerase and so prevents RNA synthesis. Ionomycin is a calcium ionophore that raises intracellular calcium levels and triggers apoptosis and staurosporine is an antibiotic from streptomyces that can inhibit protein kinase C.

Mutu BL-cells became apoptotic after an 8-hour incubation with 1μg/ml actinomycin D, 16-hours after incubation with 1μg/ml ionomycin and 8 hours after incubation with 1μg/ml Staurosporine. Mutu BL-cells were thoroughly washed in PBS and then used in a transmigration assay. All four methods effectively induced apoptosis. Mutu BL-cells induced by UVB-light were 89% apoptotic by FACS (i), when treated with actinomycin D were 85% apoptotic by FACS (ii), by ionomycin were 60% apoptotic by FACS (iii) and by staurosporine were 93% apoptotic by FACS (Figure 4.8 (iv)). Macrophages migrated preferentially to apoptotic cells compared to live cells, irrespective of the method used to induce cell death. Actinomycin D did not inhibit macrophage chemotaxis and so it is likely that Mutu BL-cells regulated the production of the "apoptotic chemokine" post-translationally, and did not transcribe it *de novo*.

Macrophage chemotaxis to apoptotic Mutu BL-cells is independent of the method used to induce apoptosis

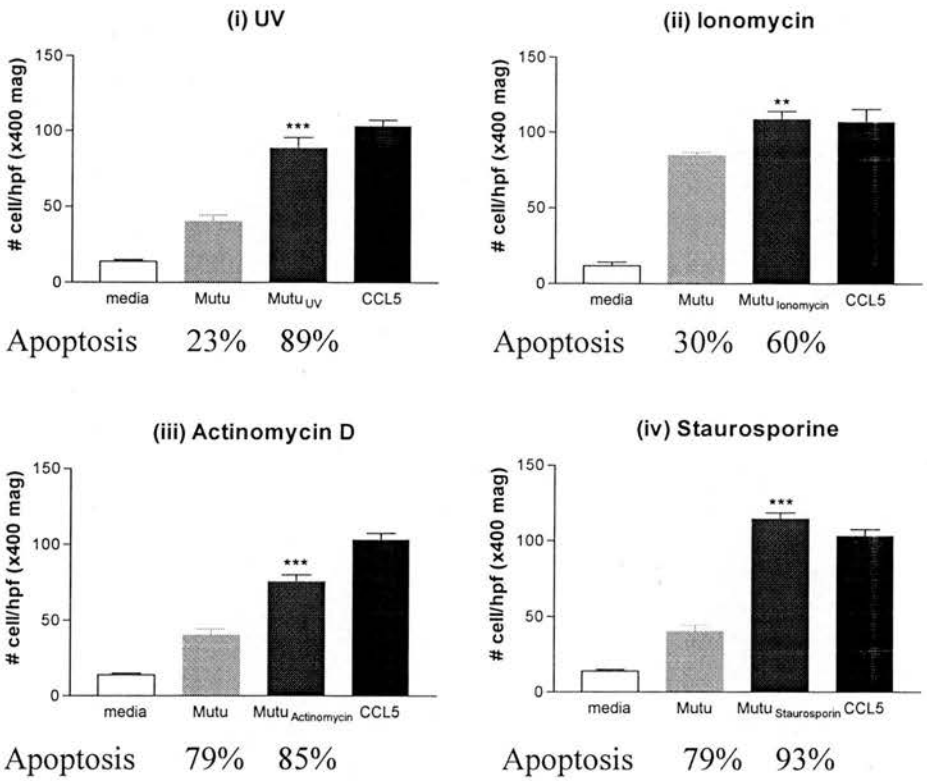


Figure 4.8

Apoptosis of Mutu was induced by 100mJ/cm2 UV-light, 1μg/ml ionomycin, 1μg/ml actinomycin D or 1μg/ml staurosporine. Macrophages were allowed to transmigrate to Mutu BL-cells, Mutu BL-cells induced into apoptosis, media only (negative control) and 100ng/ml CCL5 (positive control). The mean number (+SEM) of transmigrated cells counted per high power field for each condition was plotted.One representative result from two is shown. Macrophage chemotaxis to apoptotic Mutu was significantly higher than to live Mutu (** $p< 0.005$ and **$p< 0.0001$, student's t-test). Macrophage chemotaxis to apoptotic Mutu occurred independently of the stimulus used to induce apoptosis. % apoptosis is shown

4.9 Macrophage chemotaxis to conditioned media from apoptotic BL-cells

(Figure 4.9)

In 1999 Segundo et al published that supernatants from apoptotic germinal centre B-cells from tonsil were chemotactic for monocytes. The active chemoattractant was found in the blebs that had budded off from the apoptotic cell surface (Segundo, 1999). To determine whether chemotactic molecules from apoptotic Mutu BL-cells could be demonstrated in cell free preparations, conditioned media was prepared from $2x10^6$/ml Mutu BL-cells in serum-free media 12 hours after induction of apoptosis.

A cell-free supernatant was prepared by passing suspended cells through a sterile 0.2μm diameter filter. The ability of macrophages to transmigrate to the conditioned supernatants was compared to the original batch of $2x10^6$/ml Mutu BL-cells in suspension. Figure 9 is a representative example of three experiments and shows macrophage chemotaxis to conditioned media from apoptotic Mutu BL-cells was equal to macrophage chemotaxis to whole cells in suspension. This confirmed that the bioactive moieties were released from the apoptotic cell into the supernatants.

Subsequently, it was found that the chemoattractant activity of conditioned media began to deteriorate after storage at –20°C. Therefore, the supernatants were placed in a water bath at 95°C for half an hour to further test the stability of conditioned media. Raising the temperature to 95°C significantly inhibited macrophage chemotaxis to conditioned supernatants from apoptotic BL-cells.

It seemed likely that the heat-labile chemotactic moiety present in the conditioned supernatant was a protein because it was heat labile. This result was different to the result published by (Lauber, 2003) who had shown that conditioned supernatants from different apoptotic cell lines contained a lipid chemotactic molecule that was stable for up to ten minutes at a temperature of 90°C.

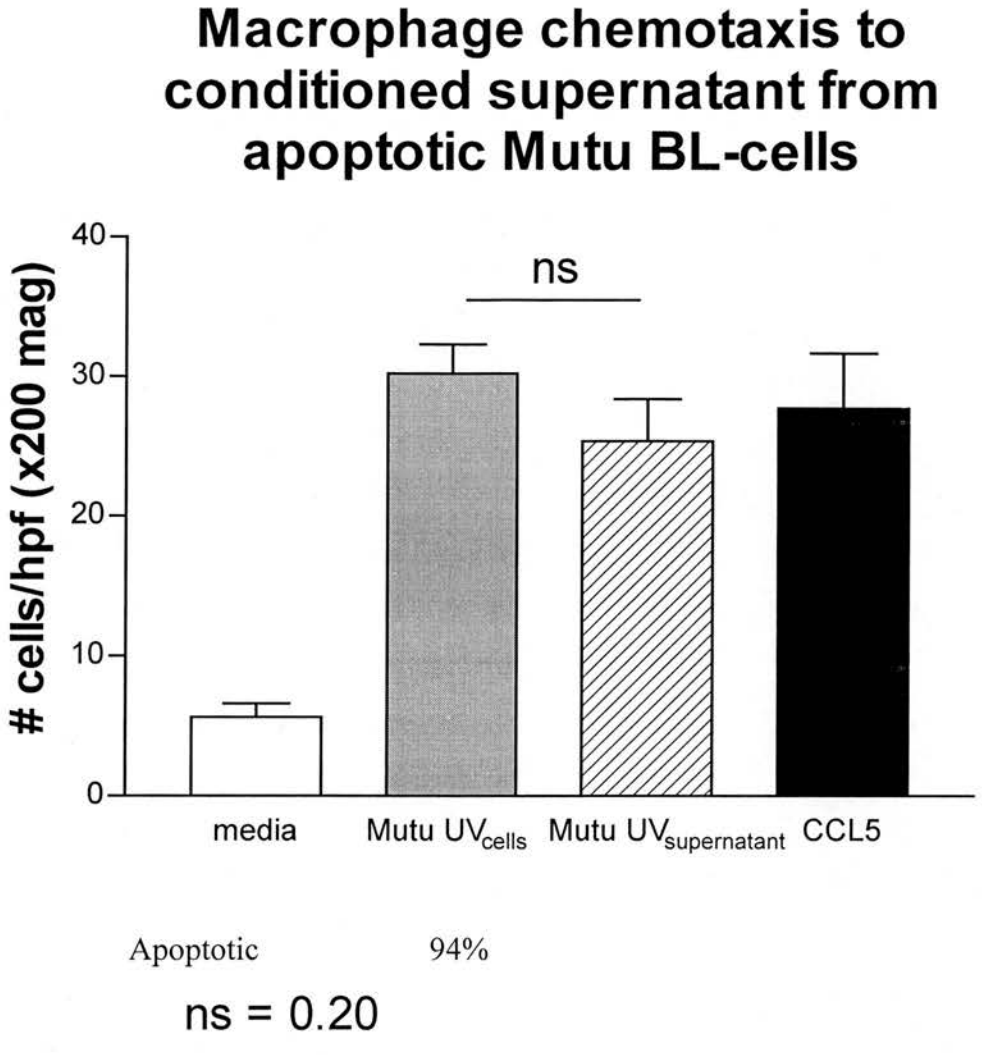


Figure 4.9

Mutu BL-cells were treated with 100mJ/cm2 UV-light to induce apoptosis.Macrophages were allowed to transmigrate to UV-treated Mutu BL-cells, the conditioned supernatant from the same cells (hatched), media only (negative control) and 100ng/ml CCL5 (positive control). The mean number (+SEM) of transmigrated macrophages per high power field for each condition was plotted.One representative result of three experiments is shown. Macrophage chemotaxis to apoptotic Mutu was significantly higher than to the negative control ($p= 0.20$, student's t-test). There was no significant difference between macrophage chemotaxis to apoptotic Mutu BL-cells, and to their cell-free conditioned supernatants. % apoptosis is shown.

4.10 Inhibitors of chemotaxis prevent macrophage transmigration to apoptotic BL-cells

(Figures 4.10a and 4.10b)

Chemokines are small protein cytokines that direct cell movement. They share common structural motifs that can be copied by certain viruses. These viral homologs can be used experimentally to inhibit cell movement. In addition, all chemokines that signal through G-protein linked receptors can have their activity altered using inhibitors of this signalling pathway.

Pertussis toxin inhibits macrophage chemotaxis to apoptotic Mutu BL-cells

(Figure 4.10a)

Pertussis toxin (PTX) was used to inhibit macrophage chemotaxis in the transwell assay. PTX blocks calcium dependent G protein-linked chemokine receptors, and could also affect macrophage chemotaxis to apoptotic Mutu BL-cells. The chemokine receptors are serpentine receptors that signal through trimeric GTP-binding proteins and so they are called G-protein-coupled receptors (GPCR) (Zlotnik, 2000). PTX catalyses the ADP-ribosylation of the alpha subunit of G_i, which in turn inhibits cAMP production by adenylyl cyclase. In such a way, PTX can have profound inhibitory effects on many GPCR including the chemokine receptors.

Overnight pre-treatment of macrophages with 100ng/ml PTX abolished macrophage chemotaxis to both apoptotic cells and CCL5 (figure 4.10a). Importantly, PTX-treated macrophages were still viable at the end of the transmigration assay: 91% of PTX–treated macrophages excluded trypan blue compared to 90% of control macrophages. However, other functions of the PTX-treated macrophages were not assessed for. With this caveat in mind, PTX inhibition of macrophage chemotaxis indicated that macrophages might be using one of 18 known chemokine receptor to move towards one of the 44 known chemokines (Zlotnik, 2000). Although chemokine receptors are notoriously promiscuous in their choice of ligand, this result helped to narrow down the search for an "apoptotic chemokine" released by apoptotic BL-cells.

Viral Macrophage Inflammatory protein II (vMIPII) inhibits macrophage chemotaxis to apoptotic BL-cells.

(Figure 4.10b)

Experiments using a viral inhibitor of chemotaxis was used to confirm that macrophages used a known chemokine receptor when transmigrating to apoptotic BL-cells. Many viruses encode homologues of chemokines and their receptors, presumably to evade immune detection (Alcami, 2003). The herpes virus that is associated with Kaposi's sarcoma (KSHV), human herpes virus 8, produces a broad-spectrum chemokine antagonist called vMIPII. Chemokine receptors from all three of the family groups (CC, CXC and CX3C) can be blocked *in vivo* by vMIPII (Kledal, 1997), although, in some situations it can be an agonist. vMIPII is chemoattractive to TH2-lymphocytes and eosinophils and can direct the type of immune response away from TH1 towards aTH2 (Weber K, S et al). Work by Chen et al, had already shown that vMIPII was able to inhibit CX3CL1-induced leukocyte migration in the rat (Chen, 1998).

Next, the inhibitory action of vMIPII on fractalkine-induced chemotaxis was examined in the human macrophage transmigration system. Macrophages were tested for their ability to transmigrate to recombinant human fractalkine in the presence of 60, 300 and 600ng/ml of vMIPII. At the lowest concentration of 60ng/ml, vMIPII was able to significantly inhibit macrophage chemotaxis to soluble fractalkine (data not shown). vMIPII had no toxic effect as to macrophages as over 90% were viable after exposure, as measured by trypan blue exclusion. Therefore, a concentration of 60ng/ml of vMIPII was used to inhibit macrophage chemotaxis to apoptotic cells.

The effect of 60ng/ml vMIPII on macrophage chemotaxis to Mutu BL-cells is shown in figure 4.10b. vMIPII was not toxic to macrophages and had no effect on chemotaxis of macrophages to Mutu BL-cells$_{bcl\text{-}2}$. vMIPII significantly blocked macrophage chemotaxis to apoptotic Mutu BL-cells (***$p<0.0001$ student's t-test).

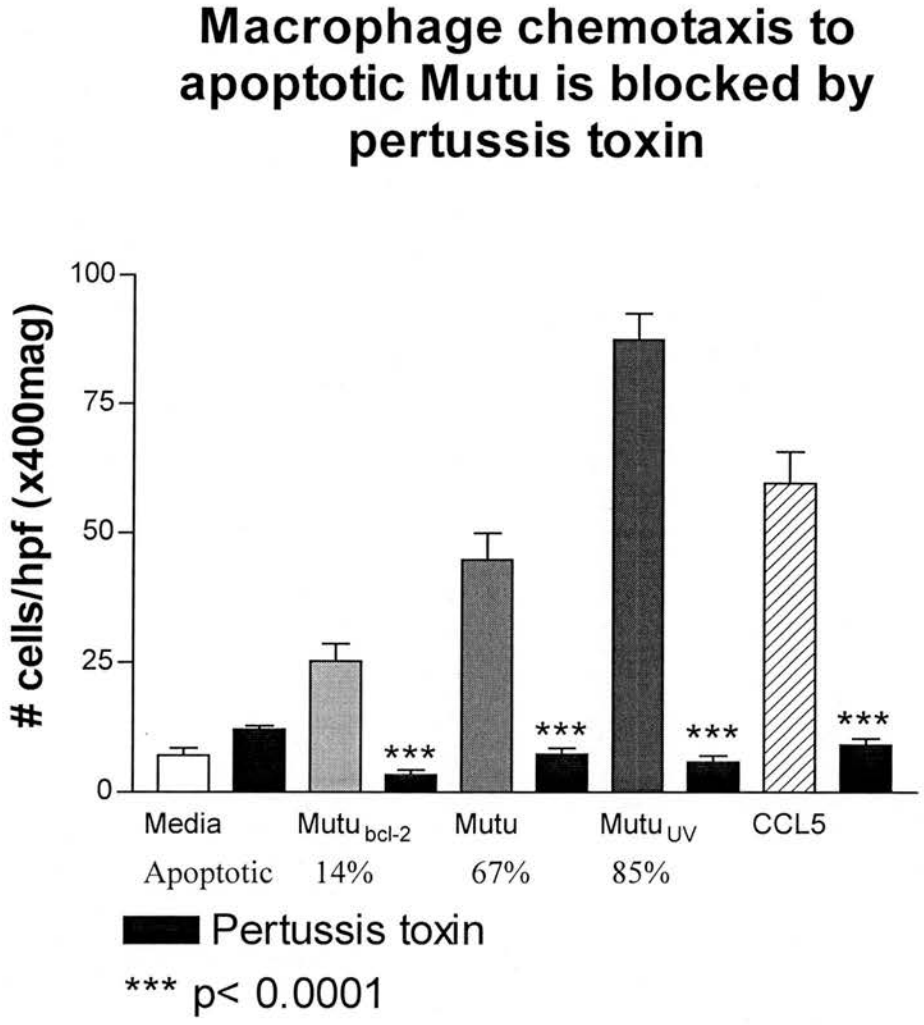


Figure 4.10a

Macrophages were treated with 100ng/ml pertussis toxin (PTX) (black bars) for 12 hours or left untreated. PTX treatment was not detrimental to macrophage viability. Macrophages were allowed to transmigrate to media alone (negative control) Mutu *bcl-2*, Mutu BL-cells, UV-treated Mutu BL-cells, or to 100ng/ml CCL5 (positive control). The mean (+SD) of macrophages transmigrating was counted for each condition. Macrophage chemotaxis to Mutu BL-cells and CCL5 was significantly inhibited by PTX treatment. (***p< 0.0001, student's t-test).% of apoptotic cells is shown.

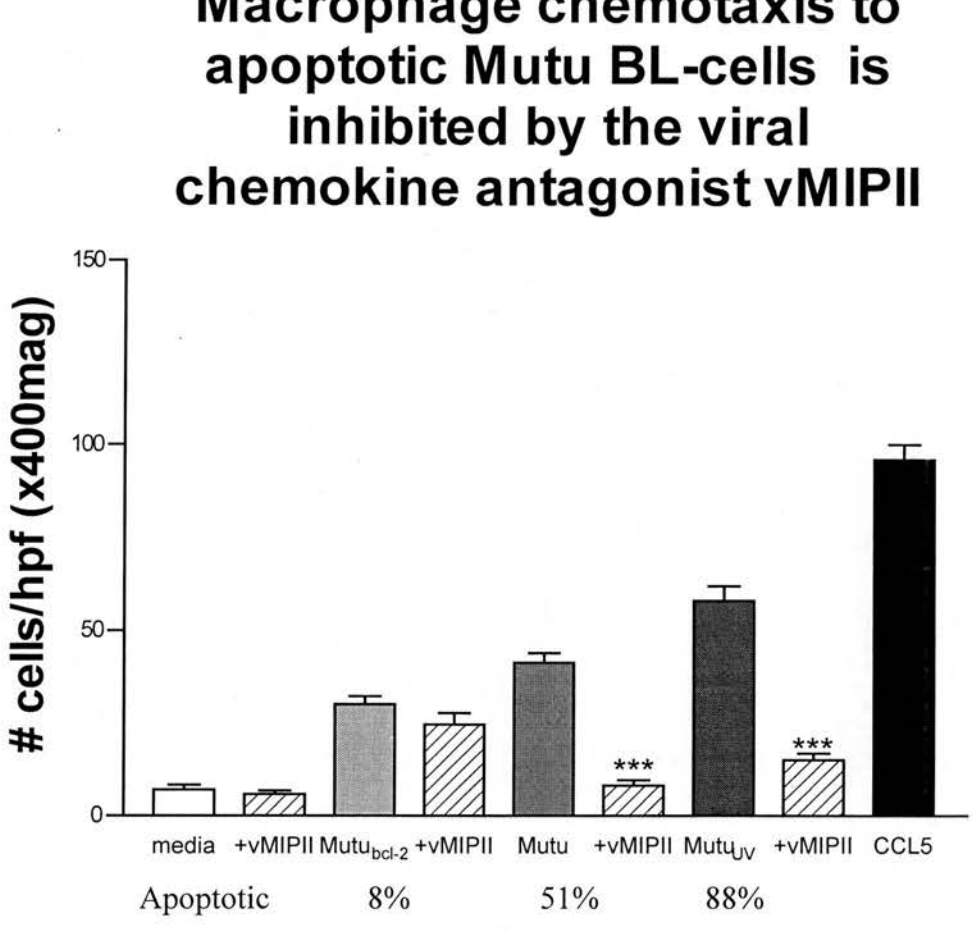


Figure 4.10b

Macrophages were allowed to transmigrate to Mutu BL-cells, Mutu BL-cells induced into apoptosis, media only (negative control) and 100ng/ml CCL5 (positive control) with (hatched bars) or without vMIPII. The mean number (+SEM) of transmigrated cells counted per high power field for each condition was plotted.One representative result of three is shown. Macrophage chemotaxis to apoptotic Mutu BL-cells was significantly inhibited in the presence of vMIPII (***p< 0.0001, student's t-test). % apoptosis is shown.

Chapter 4 summary

- Mononuclear phagocytes but not neutrophils transmigrated to apoptotic Burkitt's lymphoma cells *in vitro*. Potentially, this is a novel mechanism for apoptotic cell recruitment of tumour-associated macrophages into Burkitt's lymphoma *in vivo*.
- The lipid receptors CD14 and CD36 are not required for macrophage chemotaxis to apoptotic cells.
- Neither EBV infection nor the method used to induce Mutu BL-cells to undergo apoptosis affected macrophage chemotaxis to apoptotic cells.
- Conditioned supernatant from apoptotic Mutu BL-cells was chemotactic to macrophages and the chemotactic activity was abolished by heat treatment.
- Pertussis toxin and vMIPII blocked macrophage chemotaxis to apoptotic cells. Therefore, it is likely macrophages are using a known G-protein linked chemokine receptor.

The chemokine superfamily is now almost complete. The genome contains a complement of 42 known human ligands, all between 70-90 amino acids long and each containing two conserved-cysteine residues. Therefore it is unlikely that there are many chemokines left to be discovered (Zlotnik, 2003). Results in this chapter have suggested that apoptotic Burkitt's lymphoma cells were releasing one or more of these 42 known chemokines.

The next chapter will describe why CX3CL1 (fractalkine) was chosen as a candidate molecule for further investigation out of the 42 possible chemokines and will investigate the effect of fractalkine on macrophage chemotaxis to apoptotic cells.

CHAPTER 5

The role of fractalkine in macrophage chemotaxis to apoptotic cells

Introduction

The previous chapter showed that the viral chemokine antagonist, vMIPII, was able to block macrophage chemotaxis to apoptotic cells. vMIPII is a broad-spectrum chemokine-antagonist that blocks the actions of CXC chemokines and fractalkine (Chen, 1998). Inhibition of macrophage chemotaxis to apoptotic cells by vMIPII suggested these cells were releasing one of the 42 known chemokines that had been already characterised.

In 2003, Geissmann published work from mice describing a subset of "non-inflammatory" monocytes. These cells had high expression of the fractalkine receptor ($CX3CR1^{high}$) and constitutively homed into the lung but did not enter sites of inflammation in the gut (Geissmann, 2003). It is possible that these fractalkine-$receptor^{high}$ monocytes might also be attracted into a non-inflammatory tumour environment. In chapter 4, monocytes and macrophages, but not neutrophils, transmigrated to apoptotic BL-cells. This cell-specificity of chemotaxis to apoptotic BL-cells matched the pattern of fractalkine receptor expression (CX3CR1). I.e. Monocytes and macrophages both express CX3CR1 and move towards apoptotic BL-cells, but neutrophils do not (Imai, 1997). This suggested that the role of fractalkine in the recruitment of macrophages into Burkitt's tumours warranted further investigation.

Fractalkine had already been shown to be a direct target of p53 and as such was likely to be expressed in apoptotic cells (Shiraishi, 2000). When p53 becomes mutated, its protective effect is lost and tumours can then emerge. Mutu BL-cells have one normal p53 allele and one mutant p53 allele (Wiman, 1991). Taken together, these observations led to the hypothesis that Burkitt's lymphoma cells release fractalkine during the quiet process of apoptosis. The fractalkine produced by apoptotic cells might recruit a "non-inflammatory, $CX3CR1^{high}$" subset of macrophages into BL-tumours.

5.1 Macrophages express a functional fractalkine receptor (CX3CR1)

The original paper describing the fractalkine receptor showed that both macrophages and monocytes expressed CX3CR1 (Imai, 1997). The aim of this section was to confirm the published data and to see whether macrophages derived from peripheral blood monocytes used the fractalkine receptor in the transmigration assay *in vitro*.

Monocyte and macrophage expression of CX3CR1 mRNA

(Figure 5.1a)

RNA was isolated from the monocytic cell-lines THP-1, MM6 and primary human macrophages. RNA was reverse-transcribed and cDNA was amplified by PCR using CX3CR1-specific and actin-specific primers. Products from the PCR reaction were electrophoresed and bands of the predicted size were detected in all cell types (figure 5.1a). The products that were amplified between actin primers are shown as a positive control. Primers were designed to span intron boundaries and all samples were checked to ensure that there was no contamination of the RNA with DNA.

The results in figure 5.1a are qualitative and the bands shown can only represent the presence or absence of RNA. CX3CR1 was detected in both the THP-1 and the MM6 monocyte cell lines and in three macrophage donors. Interestingly, one of four human donor macrophages did not contain RNA for the fractalkine receptor. Next, CX3CR1 protein was quantified by specific antibody labelling, because the amount of mRNA does not necessarily reflect amounts of active protein.

Monocyte and macrophage expression of CX3CR1 protein

(Figure 5.1b)

The monocytic cell lines THP-1 and MM6, and primary human monocytes and macrophages were all immunolabelled with a rabbit-polyclonal antibody against amino acids 2-21 from human CX3CR1. A second PE-labelled goat anti-rabbit immunoglobulin antibody was used to detect bound primary antibody. A rabbit

Fractalkine receptor (CX3CR1) expression in macrophages and monocytes

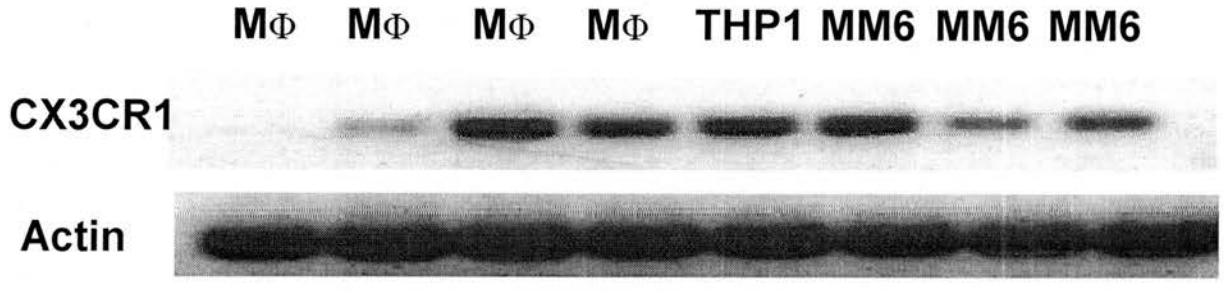


Figure 5.1a
A gel picture showing amplified products of the correct length from a RT-PCR reaction for fractalkine receptor compared to an actin control. Fractalkine receptor RNA was present in three out of four macrophage donors (MΦ) and in the monocyte cell lines THP1 and MM6. Results are not quantitative.

Fractalkine receptor (CX3CR1) expression in macrophages, monocytes, THP1 and MM6

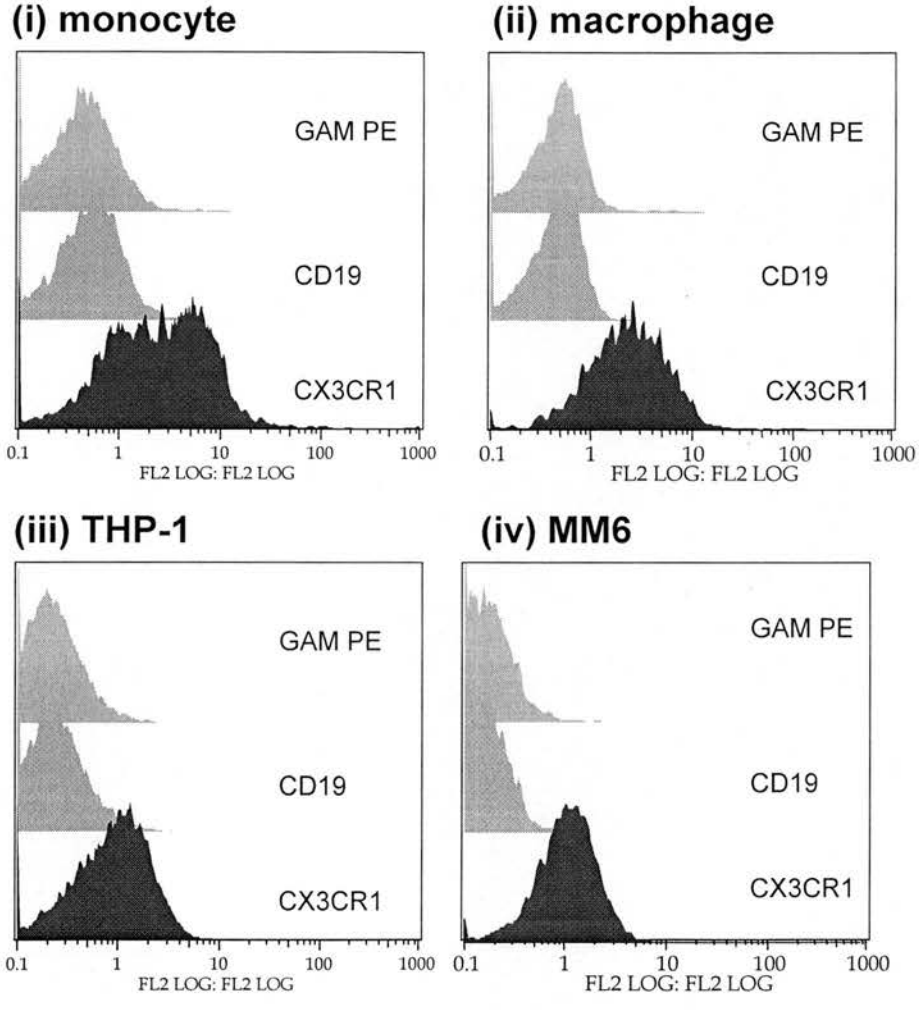


Figure 5.1b

FACS showing expression of fractalkine receptor (CX3CR1) on monocytes (i), macrophages (ii), THP-I (iii) and MM6 (iv) monocytic cell lines. Log FL2 fluorescence intensity of CX3CR1 is compared to secondary only PE labelled goat-anti rabbit Ig only or negative control CD19.

polyclonal anti-CD19 was used as a negative control because CD19 is a B-cell marker that is not expressed by monocytes.

Figure 5.1b shows log-fluorescence intensity of PE-labelled antibody staining of fractalkine receptor on THP-1 and MM6 cell lines, and monocytes and macrophages. Both monocytes and macrophages expressed the fractalkine receptor. Primary monocytes had two peaks which may represent the $CX3CR^{high}$ versus $CX3CR^{low}$ populations that have been previously described (Ancuta, 2003). Next, the functional activity of CX3CR1 was tested in the transmigration assay *in vitro*. Macrophages were initially tested for their ability to migrate to the 8.5kDa chemokine domain of recombinant human fractalkine. Overall, macrophage transmigration to recombinant fractalkine was much less than to apoptotic BL-cells. Macrophage transmigration to 50ng/ml and 100ng/ml of fractalkine was significantly greater than to media alone (data not shown), demonstrating that macrophage CX3CR1 was able to function as a chemotaxis receptor in the transmigration *in vitro* assay.

These experiments showed that primary human macrophages, derived from peripheral blood monocytes, expressed a functional fractalkine receptor. Whether Mutu BL-cells released fractalkine during apoptosis was investigated next.

5.2 Apoptotic Mutu BL-cells express fractalkine (CX3CL1)

Mutu BL-cells express fractalkine messenger RNA

(Figure 5.2a)

mRNA was isolated from Mutu BL-cells containing a range of apoptotic cells. Mutu BL-cells, which had been transfected *bcl-2* to prevent apoptosis, contained only a few apoptotic cells, whereas UVB-treated BL-cells had a higher percentage of apoptotic cells. In addition, UVB-treated Mutu BL-cells were harvested at various time-points after induction of apoptosis and mRNA was reverse transcribed. The resulting products from the PCR reaction were electrophoresed and bands of the predicted size were detected as shown in figure 5.2a. Products amplified between actin primers are shown as a positive control. The results in figure 5.2a are

only qualitative and therefore can only represent either the presence or absence of mRNA.

Live Mutu BL-cells and Mutu $_{bcl\text{-}2}$ cells with low levels of apoptosis, both expressed mRNA for fractalkine. However as soon as 2 hours after the UV-induction of apoptosis, the level of fractalkine mRNA was barely detectable and no mRNA was present at the later time points. This was not due to a general degradation of mRNA in the UV-treated cells, because the actin transcripts were still present 24-hours after the induction of apoptosis.

Actinomycin D was used to induce BL-cell apoptosis as it triggers death by preventing *de novo* mRNA transcription. The use of actinomycin D to induce apoptosis did not inhibit macrophage chemotaxis to apoptotic cells. This suggested that fractalkine mRNA may not be produced *de novo* during apoptosis but might be released pre-formed. Supporting this idea, experiments on damaged neurones had shown that membrane-associated fractalkine was released early, many hours before cell death eventually occurred (Chapman, 2000).

Mutu BL-cells express fractalkine (CX3CL1) protein

(Figures 5.2 b and 5.2c)

B cells and other leukocytes were described as having "scant" levels of fractalkine mRNA in the original paper detailing the discovery of fractalkine, (Bazan, 1997). Although subsequently, it was reported that fractalkine mRNA was expressed on activated B cells (Schaniel, 1998), fractalkine expression by apoptotic B-lymphocytes had not been investigated.

Mutu BL-cells were harvested 12 hours after UV-induction of apoptosis and the expression of fractalkine in the apoptotic cells was compared to untreated control Mutu BL-cells. It was seen that Mutu BL-cells had low levels of fractalkine staining that increased dramatically after the induction of apoptosis. When cells were back-gated onto the light-scatter plots, it was found that cells expressing the highest level of fractalkine were the leaky cells that fell into the apoptotic zone. Three different antibodies, (two mouse monoclonals (81513 and 51637.11) and one goat polyclonal) all labelled the apoptotic Mutu BL-cells in a similar pattern. Since all three antibodies labelled Mutu BL-cells in the same way, it was unlikely that they were

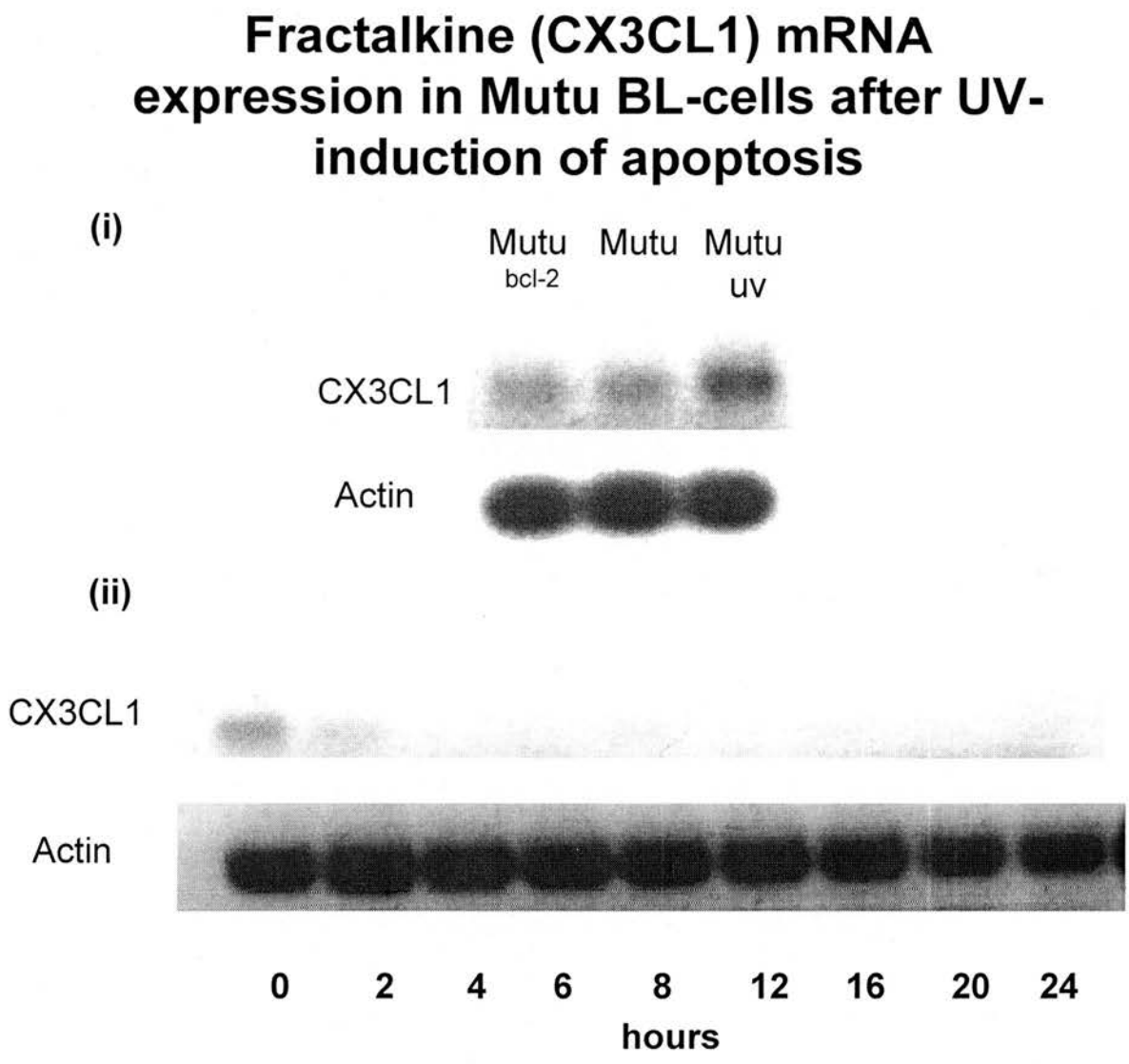


Figure 5.2a

A gel picture showing amplified products of the correct length from a RT-PCR reaction for the chemokine fractalkine (CX3CL1) compared to an actin control. Fractalkine was present in Mutu BL cells and *Mutu bcl-2* that were transfected with the anti-apoptotic *bcl*-2. RNA for fractalkine became undetectable less than 4 hours after UV-treatment to induce apoptosis. Results were not quantitative.

cross-reacting with CD84 as had been seen in previous experiments using the goat polyclonal antibody (Lucas, 2001).

To further investigate at what stage of apoptosis cells expressed fractalkine, Mutu BL-cells were harvested from a high-density culture containing a mixture of viable, early and late apoptotic cells and were triple-stained with Annexin V-FITC, Fractalkine-PE and ToPR03. Like propidium iodide, ToPr03 binds to DNA in leaky cells only after they have lost membrane integrity. Early apoptotic Mutu BL-cells were impermeable and were AxV+/ToPRO3-, whereas the late apoptotic cells were leaky and took up ToPRO3 (AxV+/ToPRO3+). FACS analysis revealed that early apoptotic cells expressed very low levels of surface fractalkine but once cells became leaky, fractalkine staining greatly increased. This suggested that fractalkine within the cells was accessible to the antibody once membrane integrity was lost during the course of apoptosis. It will be important to confirm that viable cells lose surface expression of fractalkine early in the apoptosis programme.

Fractalkine expression by apoptotic Mutu BL-cells

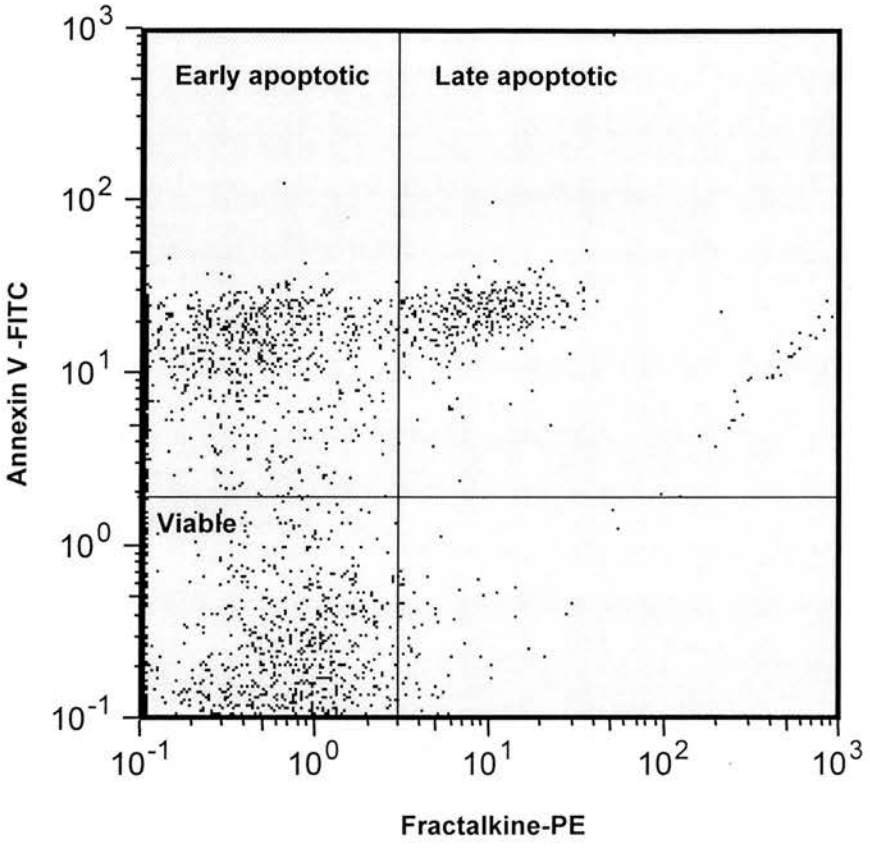


Figure 5.2b FACS dot plot of of fractalkine-PE expression by Mutu BL-cells plotted against AxV-FITC. Viable cells (AxV-/ Topr03+) express fractalkine CX3CL1. Early apoptotic cells (AxV+/ Topr03-) express less fractalkine than both viable and late apoptotic cells that are (AxV+/Topr03+).

Confocal microscopy showing antibody staining of fractalkine in Mutu BL-cells

(Figure 5.2c)

Viable and apoptotic Mutu BL-cells and were stained with either a monoclonal antibody against fractalkine or an IgG1 isotype control antibody. A secondary antibody labelled with Alexafluor-green™ was used for confocal microscopy because it was more stabile than FITC. Cells were fixed in 1% formalin prior to the addition of ToPRO3, to enable the viable cells to be discriminated from the dead cells using nuclear morphology. Under the confocal microscope, viable cells had a large nucleus surrounded by a thin rim of cytoplasm whereas apoptotic Mutu BL-cells had the characteristic "pop corn"-like nuclei.

Figure 5.2c shows a mixture of live and apoptotic Mutu BL-cells viewed under the confocal microscope. Fractalkine was labelled green and nuclei were stained blue with ToPRO3. Fractalkine was found clustered at the plasma membrane of viable Mutu BL-cells and there was no intracellular labelling. Once leaky, intracellular fractalkine was stained bright green in the apoptotic cells and also in the blebs of cell debris. These results are consistent with the notion that, following the induction of apoptosis, cells became leaky and the intra-cellular stores of fractalkine became accessible to antibody.

Western blotting of fractalkine in Mutu BL-cells

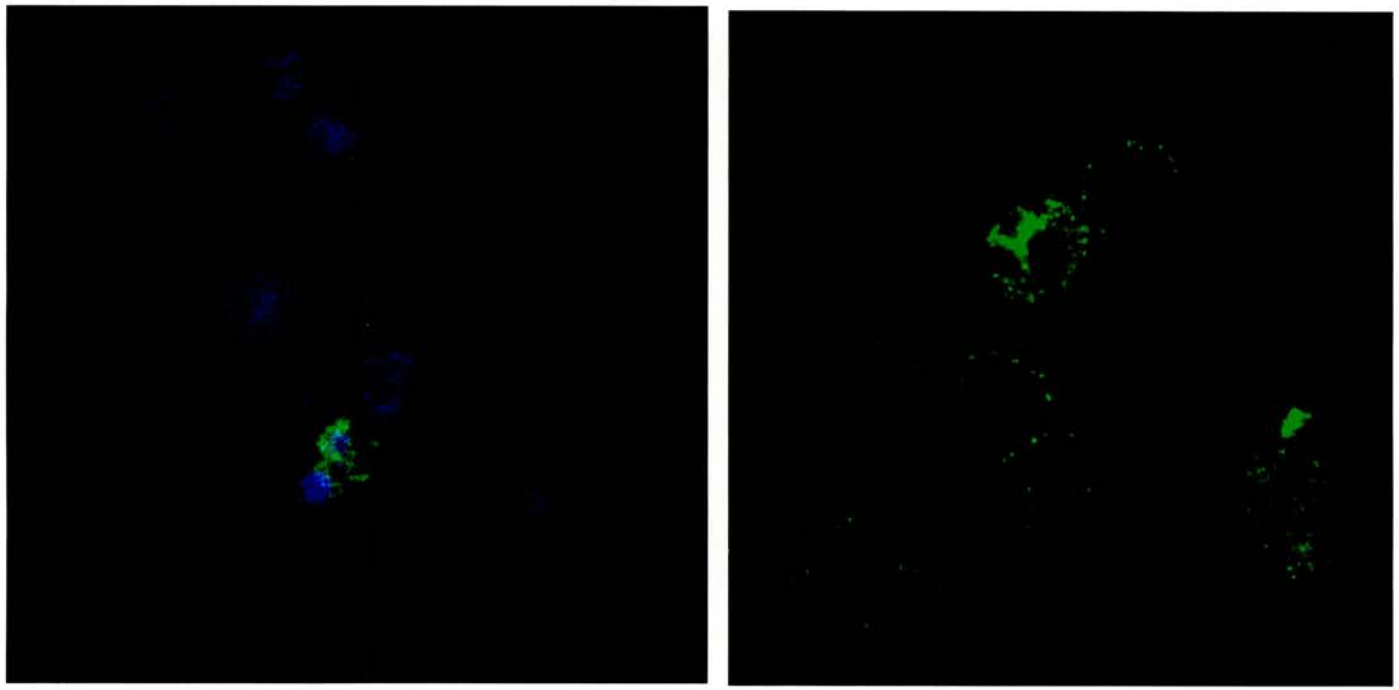
(Figure 5.2d)

Western blotting was used to further identify fractalkine in apoptotic Mutu BL-cells and to confirm the specificity of the monoclonal antibody (clone 51637.11). Cell-lysates from viable Mutu BL-cells and UVB-treated Mutu BL-cells were compared. Protein extracts were denatured and run on an acrylamide gel under reducing conditions, and the 8.5kDa chemokine domain of recombinant fractalkine was used as a positive control. The products were transferred onto nitrocellulose

Confocal microscopy of fractalkine expression in Mutu BL-cells

(i) Viable Mutu BL-cells

(ii) Apoptotic Mutu BL-cells



(iii) Mutu IgG1 isotype control

Figure 5.2
Confocal microscopy of Mutu BL-cells labelled with h ToPRO3 (blue) and fractalkine (green) in Mutu (i)and UV-treated Mutu (ii) and IgG1 isotype control (iii).

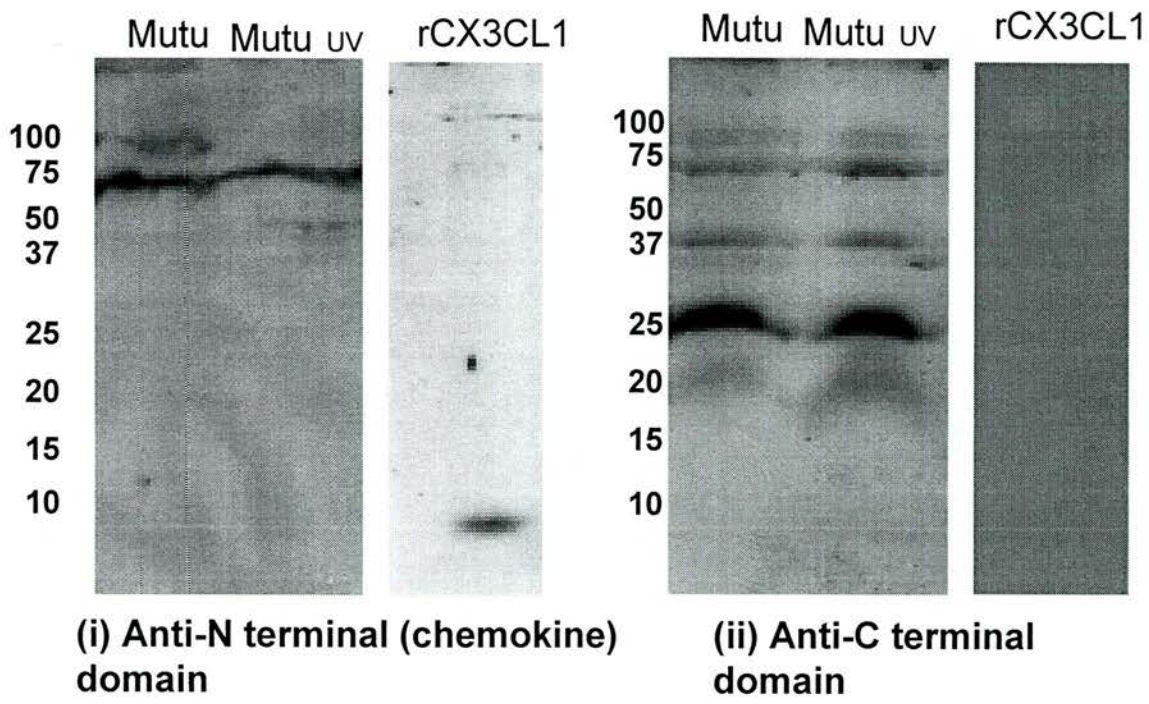


Figure 5.2d

Western blot of of Mutu BL-cell lysate, UV-treated Mutu cell-lysate and recombinant fractalkine 8.5kDa chemokine domain (positive control). Anti-N terminal 51637.11(i) and anti-C terminus antibodies (ii) labelled multiple sized bands. The predicted size of fractalkine is 60kDa and the larger 95kDa band may represent a glycosylated form of fractalkine. Smaller bands may represent cleaved fractalkine. One representative experiment of three is shown.

paper and probed with two different anti-fractalkine antibodies. Transferred proteins were labelled with either an antibody against the N-terminal (chemokine) domain (R&D systems clone 51637.11), or a chicken polyclonal antibody, (a kind gift from David Greaves, Oxford) which was raised against the C-terminal peptide of the intracellular domain of fractalkine (Lucas, 2001).

As expected, the N-terminal antibody labelled the 8.5kDa chemokine domain but the C-terminal antibody did not. The predicted size of full-length fractalkine is 95kDa. Figure 5.2f showed that the N-terminal antibody recognised two bands, one of approximately 95kDa and a second smaller band of about 60kDa. The larger band may represent glycosylation of the full-length protein. Interestingly, the larger 95kDa-band was absent in the lysates from the apoptotic Mutu BL-cells. This suggested that fractalkine was cleaved during apoptosis.

The polyclonal antibody raised against the intracellular C-terminus picked up many bands that could represent cleaved products of the full-length protein. In addition, the C-terminal antibody also picked up the same two (95kDa and 60 kDa) bands that were also labelled by the N-terminal antibody. The apoptotic cells contained different sized small lengths of fractalkine, and a dense band at 25kDa. The smaller fragments in found in the apoptotic cell lysate may be due to increased proteolysis.

Antibody-staining experiments concluded that viable Mutu BL-cells expressed full-length fractalkine on their surface that was either cleaved prior to release, or was degraded at some point during apoptosis. Further studies must be designed to measure the time-course of the release of fractalkine from the surface of cells during apoptosis. Furthermore, the detection of the secreted chemokine by ELISA of the conditioned media of apoptotic cells should be done to confirm that Mutu BL-cells release fractalkine during apoptotic cell death.

5.3 The functional activity of fractalkine in macrophage chemotaxis to apoptotic Mutu BL-cells

Having shown that Mutu BL-cells produce fractalkine, and that macrophages expressed its receptor, the ability of fractalkine to inhibit macrophage chemotaxis to

apoptotic BL-cells was tested. Fractalkine was added to the top well of the transmigration chamber in direct competition with any chemotactic molecule produced by apoptotic Mutu BL-cells in the chamber below. The anti-fractalkine neutralising antibody (clone 51637.11) was used to specifically mop up any fractalkine produced by apoptotic Mutu BL-cells in the lower well.

Fractalkine inhibited macrophage chemotaxis to apoptotic Mutu BL-cells in a competitive assay

(Figure 5.3a)

The effect of fractalkine on macrophage chemotaxis to apoptotic Mutu BL-cells was tested in a competition assay in which 100ng/ml of the recombinant chemokine domain of fractalkine was added to the top chamber (that contained the macrophages), for the duration of the transmigration assay. This approach was taken to determine whether chemotaxis towards fractalkine produced by Mutu BL-cells could be prevented by effectively abolishing the chemotactic gradient. Figure 5.3a shows that fractalkine significantly inhibited macrophage chemotaxis to media, to Mutu BL-cells and to apoptotic Mutu BL-cells ($*p<0.05$ and $***p<0.0001$ respectively, student's t-test). However, the addition of fractalkine to the upper well did not affect macrophage chemotaxis to 100ng/ml CCL5, which was the positive control.

The fact that fractalkine blocked chemotaxis to Mutu BL-cells but not to CCL5 suggested that soluble recombinant fractalkine was specifically competing with the fractalkine released by the apoptotic cells below. However, results showed that there was also an element of non-specific inhibition of macrophage chemotaxis to the media. It was possible that when fractalkine was placed in the upper well with macrophages, it was able to de-sensitise its own receptor, perhaps by down-regulating its surface expression.

It was probable that there was an element of non-specific inhibition that caused fractalkine to inhibit macrophage chemotaxis to the media (negative control). Therefore, to overcome this problem, a monoclonal antibody that could neutralise fractalkine was used to examine the specific contribution of fractalkine to macrophage chemotaxis to apoptotic Mutu BL-cells.

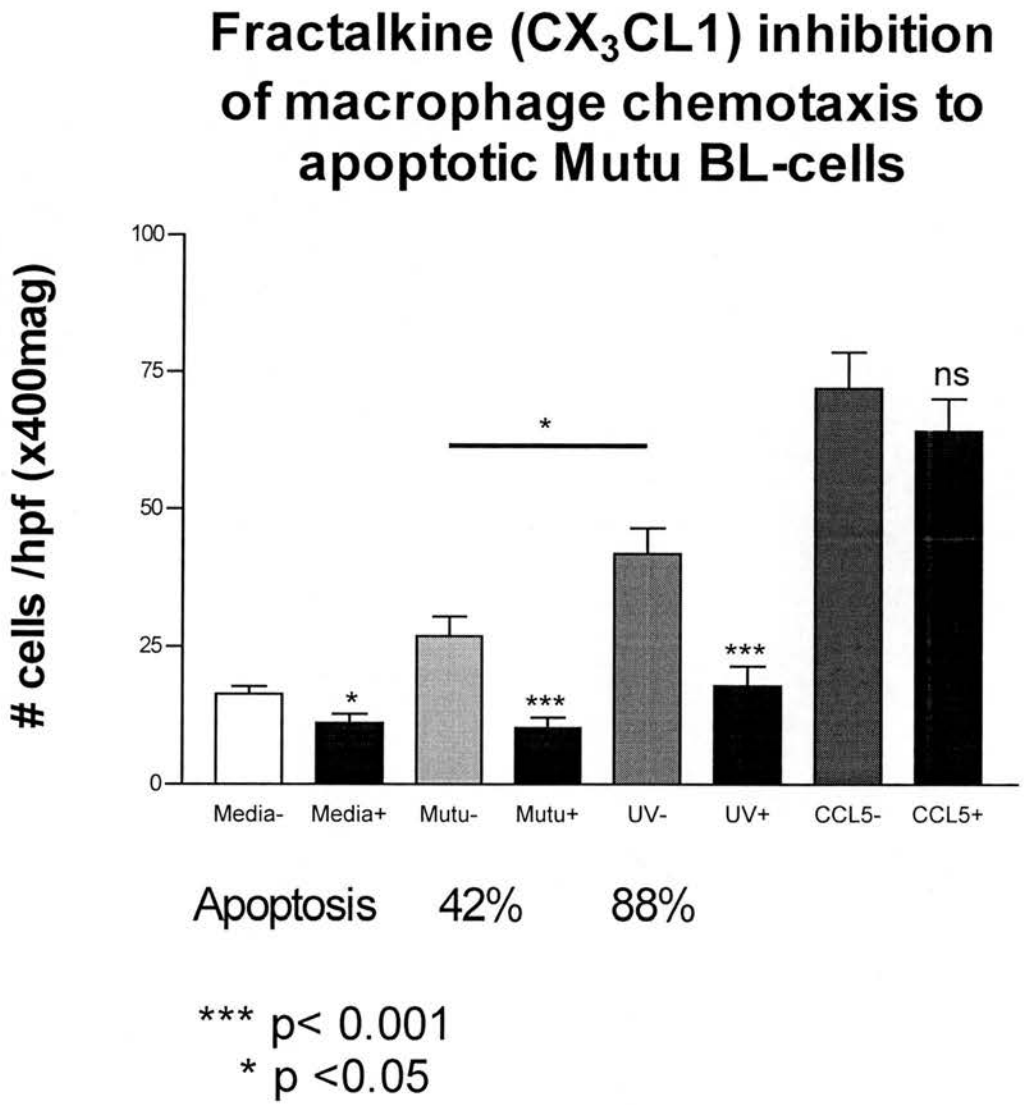


Figure 5.3a

Macrophages were placed in a transwell with or without 100ng/ml of fractalkine and were allowed to transmigrate to media alone (negative control), Mutu, UV-treated (apoptotic) Mutu and 100ng/ml CCL5 (positive control). One representative experiment of six is plotted, the mean + SEM number of macrophages that transmigrated was counted for each condition. % of apoptotic cells is shown. Macrophage chemotaxis to media, Mutu and apoptotic Mutu was significantly inhibited by fractalkine in the upper well (*$p<0.05$ (media),***$p<0.001$ Mutu and Mutu UV, student's t-test).

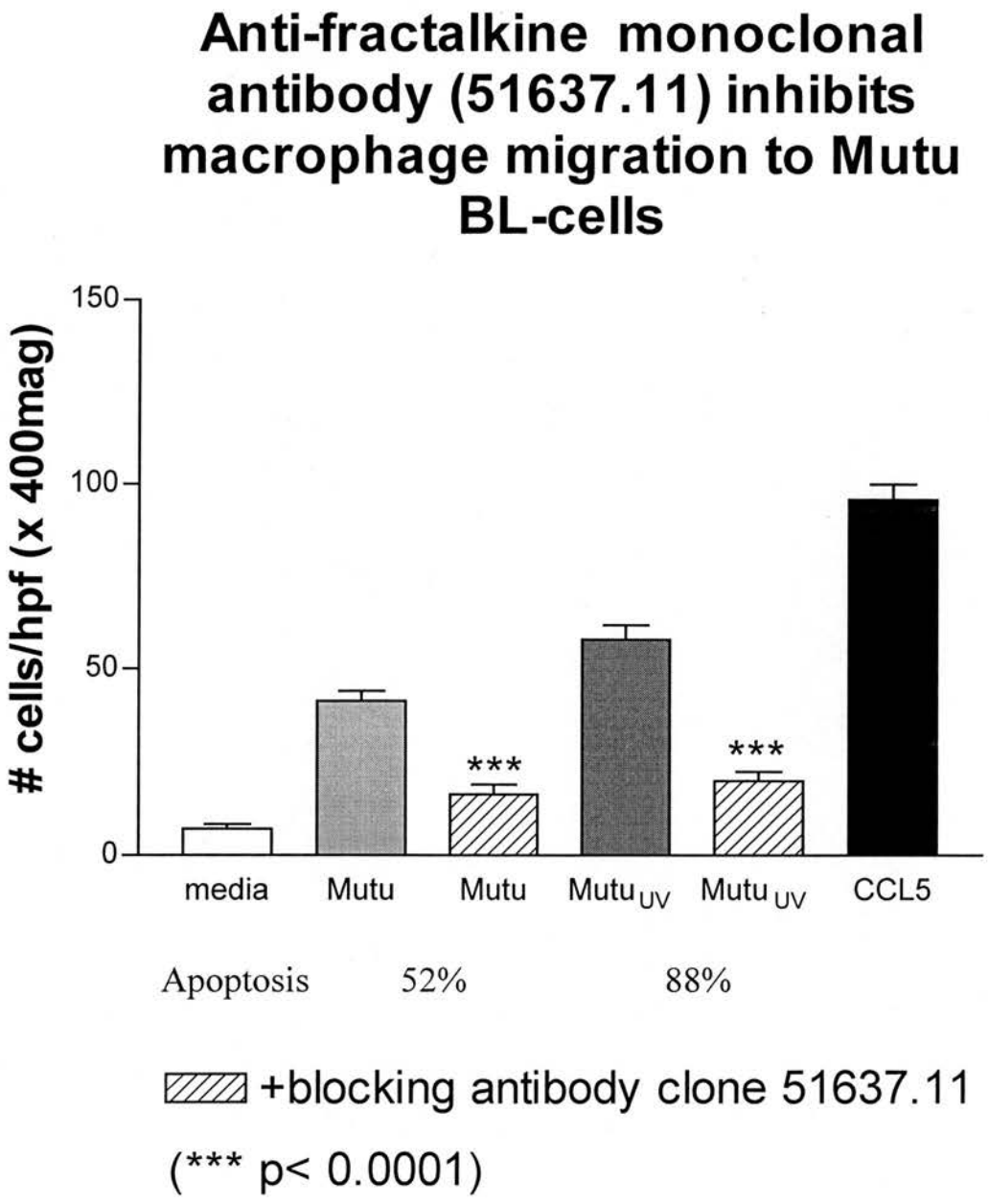


Figure 5.3b

Macrophages were allowed to transmigrate to media alone (negative control), Mutu, UV-treated (apoptotic) Mutu and 100ng/ml of CCL5 in the presence or absence of a blocking monoclonal antibody against the chemokine domain of fractalkine (clone 51637.11). One representative of four experiments is plotted and the mean (+SEM) number of macrophages that transmigrated is shown for each condition. Macrophage chemotaxis to Mutu and apoptotic Mutu was significantly inhibited by 50μg/ml of the antibody when placed in the lower well (***$p<0.0001$, student's t-test).

Anti-fractalkine antibody (clone 51637.11) blocks macrophage chemotaxis to apoptotic Mutu BL-cells

(Figure 5.3b)

A monoclonal antibody was used o determine how important the contribution of fractalkine was to macrophage chemotaxis to apoptotic Mutu BL-cells. The mouse monoclonal anti-fractalkine antibody (clone 51637.11) binds to the chemokine domain of fractalkine and blocks its action. First, the antibody was tested to determine its ability to inhibit macrophage migration to recombinant fractalkine.

50μg/ml of 51637.11 was added to the lower well of a transmigration assay in order to block the chemokine activity of any fractalkine released by Mutu BL-cells. Figure 5.3b shows that 51637.11 was able to block macrophage chemotaxis to Mutu BL-cells. The inhibitory effect of 50μg/ml of the 51637.11 antibody was compared to an IgG1 isotype control antibody at the same concentration. Again, the anti-fractalkine antibody reduced macrophage chemotaxis to apoptotic Mutu BL-cells, whereas the isotype control antibody had no effect. In all of these experiments, the anti-fractalkine antibody only partially blocked macrophage migration. This may have been because other chemotactic molecules, besides fractalkine, were being released from apoptotic Mutu BL-cells or that the concentration of the antibody was not saturating. It is unlikely that fractalkine is the sole chemokine responsible for macrophage migration, because other chemotactic molecules like the phospholipid LPC are also released by cells during apoptosis (Lauber, 2003).

Chapter 5 summary

This chapter has shown:

- Macrophages express a functional receptor for fractalkine (CX3CR1)
- Live and apoptotic Burkitt's lymphoma cells express fractalkine
- An anti-fractalkine neutralising antibody was able to inhibit macrophage chemotaxis to apoptotic Burkitt's lymphoma cells

CHAPTER 6

Discussion

This thesis has investigated the role of macrophage receptors in the chemotaxis to, and engulfment of apoptotic cells. The work presented here sprung from the observation that the CD14 knockout mouse had accumulated large numbers of apoptotic cells in its organs. Antibodies against CD14 blocked human macrophages from phagocytosing apoptotic cells, and revealed for the first time, CD14's function in the clearance of apoptotic cells (Devitt, 1998). It followed from this observation, that mice deficient for the CD14 receptor might also have a defect in the phagocytosis of apoptotic cells.

Further work presented in Chapter 3 of this thesis, identified a strain-specific defect in the phagocytosis of apoptotic lymphocytes *in vitro* by the BALB/c CD14-/- macrophages. CD14 is used by macrophages to grasp apoptotic cells before phagocytosing them. It is not known if the tethering defect reduced the efficiency of apoptotic cell-clearance and was responsible for the accumulation of apoptotic cells seen in the BALB/c CD14-/- mouse *in vivo* (Devitt, 2004). Previous work by the group observed that different tissues in the CD14-/- had excess numbers of apoptotic cells that were "free" from contact with surrounding macrophages. This suggested that the CD14-/- macrophages might also have a difficulty "sensing" and "moving towards" dying cells in addition to the observed phagocytosis defect. While much has been learned of the mechanisms by which apoptotic cells are recognised and engulfed by macrophages, little is yet known of the processes underlying the 'sensing' of apoptotic cells by phagocytes. Recent studies have highlighted the importance of chemokine release during normal cell death, and of how macrophages may sense and then move towards a dying cell in readiness to engulf the apoptotic corpse (Audran, 1996; Kao, 1994; Lauber, 2003; Cocca, 2002; Horino, 1998; Vegundo, 1999).

The fact that the CD14-/- mouse macrophages had failed to tether apoptotic cells might be one explanation for the accumulation of apoptotic cells seen in the thymus. So, the importance of CD14 in human macrophage clearance was investigated next in a subset of monocytes that expressed low levels of CD14. This minority subset of circulating human monocytes was originally isolated from peripheral blood mononuclear cells and expressed low levels of both surface CD14 ($CD14^{low}$) and CD16 (Ziegler-Heitbrock, 1991). Experiments described in Chapter 3

showed that $CD14^{low}$ monocytes maintained low levels of surface CD14 over 7 days in culture during which they matured into macrophages. Similar to mouse CD14-/- macrophages, the human $CD14^{low}$ macrophages were also found to be inefficient at interacting with apoptotic cells.

Earlier studies on the $CD14^{low}$ monocytes had shown that they expressed a different set of chemokine receptors compared to the $CD14^{high}$ cells (Weber, 2000). The $CD14^{low}$ monocytes expressed high levels of CX3CR1 and responded to its chemokine, fractalkine (Ancuta, 2000). It was also possible that CD14 might be important for reading the "come-get me" signals put out by apoptotic cells. The $CD14^{low}$ monocytes are a subset of cells with distinct migrating characteristics. Human $CD14^{low}$ monocytes display a specific set of chemokine receptors, as well as the fractalkine receptor (CX3CR1) they also express CCR5 (Ancuta, 2003; Geissmann, 2003; Weber, 2000). Study of human $CD14^{high}$ monocytes showed that they expressed CCR2 and moved towards CCL1 (MCP-1) (Weber, 2000). Whereas the $CD14^{low}$ monocytes failed to respond to CCL1, but moved preferentially to CX3CL1 (fractalkine) instead (Ancuta, 2003). The $CD14^{low}$ $CX3CR1^{high}$ monocytes are expanded in HIV and may promote infection because CX3CR1 itself is a co-receptor for the virus (Thieblemont, 1995). The migratory patterns of these $CD14^{low}$, $CX3CR1^{high}$ monocytes seem to be quite distinct from the $CD14^{high}$ monocytes. Expression of the fractalkine receptor was able to direct mouse monocytes away from the site of peritoneal inflammation and into the lungs (Geissmann et al., 2003). This seemed to mark out a different, kind of role for these $CD14^{low}$, $CX3CR1^{high}$ monocytes.

Every day, about 340 million monocytes leave the circulation in the absence of any inflammation at all. However, the steady-state mechanism that controls this enormous exodus of cells is largely unknown (Muller, 2001). It remains to be seen whether the same chemokines that are released during inflammation, can also recruit monocytes into unperturbed tissues. One or two, so-called "constitutive" or "homeostatic" chemokines, like CCL20 (SLC) and CXCL14 (BRAK), have been described, but none of these are widely or consistently expressed throughout the body (Kurth, 2001). Fractalkine receptor (CX3CL1) may be a candidate for a "constitutive" chemokine that directs monocyte traffic (Lucas, 2003; Geissmann,

2003; Kremlev, 2005). Fractalkine is widely expressed in the body for e.g. by epithelial cells and neurons, and its receptor is found on both monocytes and macrophages (Jung, 2000; Bazan, 1997; Lucas, 2001). To maintain the number of resident tissue macrophages there must be a constant recruitment of monocytes. Therefore a constitutive chemotactic molecule might be released during normal cell turnover. Since apoptosis occurs in all tissues, a dying cell may be one source of such a chemotactic molecule that could recruit monocytes destined to become the next tissue macrophages. Once in situ, these tissue macrophages could themselves release growth factors to stimulate stromal cell division (Chazaud, 2004).

Supposing that apoptosis was an engine driving the constitutive migration of monocytes out of the circulation and into tissues, it might also direct macrophages into tumours that contain frequent apoptotic cells. The work presented in this thesis shows that fractalkine (CX3CL1), a chemokine that is known to direct the homeostatic flow of $CX3CR1^{high}$ monocyte traffic into tissues like the lung, may indeed be important in recruiting macrophages to a non-inflammatory tumour environment. Results presented here show that apoptotic Burkitt's lymphoma cells expressed fractalkine that could attract monocytes and macrophages towards tumours cells *in vitro*. These observations increase our understanding of macrophage chemotaxis to apoptotic cells and may shed light on the recruitment of tumour-associated macrophages into Burkitt's lymphoma.

6.1 The persistence of apoptotic cells may sometimes be anti-inflammatory

Although CD14 is important for tethering apoptotic cells prior to phagocytosis (Devitt, 1998; Devitt, 2004), the results presented here demonstrate that it is not required for macrophage chemotaxis. Experiments with BMDM showed that CD14-/- Balb/C mouse-macrophages had no defect in migration to apoptotic cells. However, when human macrophages were used in the transmigration assay, the cells that had migrated expressed high levels of CD14. Perhaps this CD14 was upregulated in readiness to tether and engulf an apoptotic target. Unexpectedly, the apoptotic cells so prominent in the CD14 knockout mice persisted in the absence of inflammation or autoimmune disease, even into ripe old age (Devitt, 2004). Put

together, this suggests that rather than being pro-inflammatory, persistent apoptotic cells may well sustain an anti-inflammatory environment, at least in the CD14 knockout mouse and perhaps also in a tumour situation (Ogden, 2005).This work supports a role for apoptotic cells in tumour survival. Furthermore, the persistence of apoptotic cells may even aid wound healing, neovascularisation and promote tissue repair (Sun, 2005; Volin, 2001). Experiments from the CD14 -/- mouse support a rather controversial view that challenges the dogma that necrotic cells are necessarily pro-inflammatory (Gregory, 2004).

Interactions between phagocytes and apoptotic cells can be neutral but they can also dramatically suppress inflammation. This is usually achieved by secretion of cytokines like TGF-β, PGE_2, and PAF (Voll, 1997; Fadok, 1998; Fadok, 1998; Kurosaka, 2003). The view that necrotic cells and persisting apoptotic cells are inflammatory is challenged by the apparently harmless accumulation of apoptotic cells seen in the CD14 knockout mouse and also described in this work (Devitt, 2004 ; Gregory, 2004; Bryne, 2002). Although macrophage interactions with apoptotic cells are often non-inflammatory or even anti-inflammatory, there are some exceptions to this. Indeed, apoptotic cells can be inflammatory. Repeated injection of apoptotic cells leads to auto-antibody production, leading to the production of auto-antibodies and immune diseases like SLE (Botto, 1998; Scott, 2001; Cohen, 2002; Szondy, 2003; Hanayama, 2004; Casciola-Rosen, 1994; Mevorach, 1998). Impaired phagocytosis of apoptotic cells leads to autoantigens being cross-presented by DCs to CD8 T cells causing diseases like SLE (Albert, 1998; Albert, 1998a). Dying cells also release uric acid that is a potent adjuvant that can stimulate lymphocyte responses (Gallucci, 1999; Shi, 2003). Therefore, the dogma that apoptotic cells are "good" and necrotic cells are "bad," appears to be simplistic (Gregory and Devitt, 2004).

The observation that CD14-/- mice had persistent apoptotic cells in the absence of inflammation is striking, and needs to be further examined using different background strains. This work described an accumulation of apoptotic cells in the thymus in the BALB/c strain that was not apparent in the C57BL/6. In light of recent, strain-specific differences seen in the phenotypes of the PSR-/- mice, the CD14 knockout needs careful examination on the C57BL/6 background (Weitzman, 2004;

Kunisaki, 2004; Böse, 2004). It will be interesting to discover whether the C57BL/6 CD14-/- also has a phagocytosis defect *in vitro*. It may be that the C57BL/6 strain does not rely so heavily on CD14 for apoptotic cell removal. The C57BL/6 may use an alternative repertoire of phagocytic receptors to the BALB/c. The clearance mechanisms in the C57BL/6 might need to be stressed by injury or infection, mimicked by using steroids *in vitro*, to induce overwhelming apoptosis that then exposed a clearance defect.

For this work the different mouse strains were housed in different conditions; the C57BL/6 were kept in specific pathogen-free cages whereas the BALB/c were in conventional housing. Arguably, these different environments may lead to differences in the presence of low-grade infection or in commensal flora. Any infection (how ever insignificant) might increase cell apoptosis or reduce the effectiveness of macrophage clearance. The original paper that described the CD14-/- knockout showed these mice had a paradoxical rise in the number of bacteria surviving in the blood (Haziot, 1996). Additionally, neutrophil infiltration was delayed into the peritoneum of infected CD14-/-animals (Yang, 2002) suggesting that an inflammation caused by infection may not be apparent. In these circumstances it would be possible to culture organisms directly from blood or perform PCR to completely rule out the presence of such sub-clinical infections. However, CD14-/- mice had no defect in the phagocytosis of group B streptococci or IgG-opsonised cells (Haziot, 1996; Henneke, 2002; Devitt, 2004). It is conceivable that loss of CD14 may delay the evolution of the innate response, and it follows that macrophages deficient in CD14 may not always produce the cardinal signs of inflammation. However BALB/c CD14-/- animals seemed to show no systemic signs of ill health, they produced TNFα responses to LPS and had normal auto-antibody titres; in addition, CD14-/- macrophages produced TGFβ after engulfing apoptotic cells (Devitt, 2004). The clearance mechanisms available to macrophages are so varied and efficient that it is rare to observe apoptosis on routine histological slides. Apoptotic cells are rarely seen in the histological analysis of normal tissues but are more common in embryonic tissues and in developing thymus, were cell-turnover is massive. Therefore, an infection needs be massive in order to overcome this clearance system. The apoptotic cells that are so visible in the CD14-/- mouse

thymus are more likely to be due to clearance defect to apposed to a sub-clinical infection.

The increased numbers of apoptotic cells observed in the thymus of the CD14-/- mice may reflect a failure of thymocyte selection and that the loss of CD14 would also affect antigen presentation in the thymus (Esashi, 2003). The fact that apoptotic cells are found widely distributed in the BALB/c CD14-/- mouse, and not just in the thymus again points to a genearalised clearance defect rather than to a change in the level of T cell survival in these mice. It is interesting to note that the BALB/c CD14-/- mouse also had increased numbers of apoptotic cells in non-lymphoid organs like the lung and colon (Devitt, 2004). This widespread distribution of apoptotic cells is not seen in all knockout-mice deficient for other phagocytic receptors. For example, the C1q-/- mouse has an organ-specific accumulation of apoptotic cells in the kidney but not in the spleen (Botto, 1998). Overall, the persistence of apoptotic cells in the CD14-/- appears to have no deleterious effect on the mouse, and likewise, as will be discussed below, the apoptotic cells in Burkitt's lymphoma may promote tumour evolution and prolong tumour life

6.2 Are macrophage phagocytic receptors also important for chemotaxis to apoptotic cells?

Many of the receptors involved in apoptotic cell clearance, including CD14 and scavenger receptors, have been seconded from other duties, like scavenging lipids and immunological tasks. It is possible that one or more of the many phagocytic receptors has a second role: that of finding or sensing, an apoptotic cell. Chemotaxis is the directional movement of a cell up a chemokine gradient and is important in a number of biological processes including leukocyte recruitment to sites of inflammation, and wound healing (reviewed in Zlotnik, 2000). The work presented here reveals an additional role for fractalkine in macrophage chemotaxis to apoptotic cells. Different groups have earlier described a variety of chemotactic molecules (such as PAF, LPC, S19 and EMAPII) released by apoptotic cells (Bratton, 1993; Horino et al., 1998; Wakasugi, 2003; Lauber, 2003; Kao, 1994). Until now however, no one has yet demonstrated the release of a known chemokine by apoptotic cells

The migration of macrophages to "come-get-me" signals from apoptotic BL-cells was examined in Chapter 4. Migration and phagocytosis involve common membrane and cell shape changes and both processes are known to be regulated by the same signalling pathways involving integrins and small G proteins (Hogg, 2001; Aderem, 1999). Indeed, apoptotic-cell engulfment and cell migration in the nematode worm *Caenorhabditis elegans* have been shown to be governed by common genes that are conserved in flies and mammals (Wu, 1998). Recent experiments in *C. elegans* show that the intracellular pathways of chemotaxis and engulfment come together resulting in lamellipodia formation (deBakker, 2005). The upstream membrane receptor that recognises the "come-get-me" and "eat-me" signals may well be one of approximately 550 G-protein-coupled chemoreceptors present in the worm's genome (Robertson, 1998).

Given the mechanistic similarities between phagocytosis and chemotaxis, it was supposed that macrophage receptors involved in the recognition and engulfment of apoptotic cells might also have a dual function in the chemotactic response of macrophages to such cells. Not only would these macrophage receptors recognise apoptotic cell's "eat-me" ligands but they may also pick up "find-me" clues. The discovery that apoptotic cells released the lipid lysophosphatidylcholine (LPC) supported the investigation of the phagocytic lipid-receptors CD14, and CD36 in chemotaxis of macrophages to dying cells (Lauber et al. 2003). Although a dying cell may well release lipids to attract phagocytes towards the end of its life, the results from this work show that macrophage chemotaxis to an apoptotic cell does not require the 'phagocytic lipid receptors' CD14 and CD36. The negative results from this part of the work do not invalidate the general approach. If the search had continued it may well have proved fruitful and have led to the discovery of the involvement of a chemokine receptor like fractalkine. Later on during the course of these studies the similarity in the structure of CD36 and the chemokine CXCL16 was the clue that fractalkine might be a candidate chemokine that was released by apoptotic cells (Shimaoka, 2000; Shimaoka, 2004; Wuttge, 2004).

CD36 is a class B scavenger receptor that binds oxidised lipids and is an important contributor to the pathogenesis of atherosclerosis. In addition to binding native lipids, CD36 can also recognise the PAMP lipoteichoic acid, produced by

gram-positive bacteria (Hoebe, 2005). SR-PSOX is a scavenger receptor, expressed by macrophages that is also found in atherosclerotic lesions (Minami, 2001). The surprising finding about SR-PSOX was that it was also a chemokine and was given the name CXCL16 (Wuttge, 2004). Subsequently, quite a number of chemokines have been identified that also have a scavenger function (Shimaoka, 2004). Although CXCL16 and fractalkine belong to different chemokine families they both have the same "chemokine on a stalk" structure. Despite this similarity, no scavenger function for fractalkine has been identified so far. However, fractalkine may bind to an unidentified PAMP on red blood cells infected by Plasmodium falciparum. The sequestration of parasitised erythrocytes in cerebral vessels is a lethal development in the pathogenesis of malaria and the same antibodies that prevented macrophage chemotaxis to apoptotic cells blocked this binding (Hatabu, 2003). The fact that Plasmodium falciparum, an organism that has evolved to live undetected in its host, interacts with fractalkine is very interesting. The parasite may have used fractalkine to provide a non-inflammatory conduit into the host cells. It is also possible that the parasite is camouflaging itself as an effete erythrocyte to take advantage of the quiet immune response associated with the clearance of apoptotic cells.

Interestingly, the surface of parasitised erythrocytes contains "knobs" that mediate the adhesion of the parasitised cells under flow conditions. The malaria parasite-protein PfEMP-1 is concentrated on these knobs and is able to bind to CD36, and this interaction can be blocked by PS-containing liposomes (Eda, 2002). The knobs on parasitised cells carrying motifs recognised by both CD36 and fractalkine look very similar to the blebs seen on apoptotic cells. During apoptosis a cell pinches off small "blebs" of its plasma membrane. On the blebs are concentrated autoantigens, and possibly chemoattractant molecules that provide a chemotactic gradient for macrophages to trace to an apoptotic cell (Casciola-Rosen, 1994; Segundo, 1999).

6.3 Is fractalkine a non-inflammatory chemokine?

This work describes the release of fractalkine by apoptotic Burkitt's lymphoma cells and the possibility arises that fractalkine might contribute to the anti-inflammatory milieu of the tumour. This may be surprising, given that early work

grouped fractalkine with inflammatory chemokines. The labelling of fractalkine as inflammatory must be revisited in light of the work presented here and other recent publications (Lucas, 2003; Geissmann, 2003; Kremlev, 2005).

Much evidence supports a pro-inflammatory role for fractalkine (Efsen, 2002; Fraticelli, 2001; Nishimura, 2002; Yoneda, 2000; Yoshida, 2001). The "chemokine on a stalk" structure of fractalkine allows it to both attract and tether leukocytes to endothelium (Haskell, 2000; Bazan, 1997). An anti-fractalkine antibody could prevent monocyte infiltration of the synovium in collagen-induced arthritis, but did not increase production of IFNγ or auto-antibody (Nanki, 2004). TNFα together with IFNγ together raised fractalkine levels in astrocytes and smooth muscle cells (Yoshida et al., 2001; Ludwig, 2002). Whereas IL-4 and IL-13, both TH2-type cytokines have no effect on fractalkine expression. This has lead to a model of fractalkine being able to polarise T cells towards TH1-type responses (Fraticelli et al., 2001). Fractalkine is also produced during liver injury and anti-fractalkine antibodies can prolong the survival of cardiac allografts (Efsen et al., 2002; Haskell, 2001); the CX3CR1-expressing cells doing the damage to these organs are cytotoxic lymphocytes and NK cells.

The majority of work to date describe fractalkine's major role as inflammatory, but some more recent publications reveal a more complex, reparative and non-inflammatory role for this cytokine (Lucas, 2003; Geissmann, 2003; Kremlev, 2005). This would fit in with fractalkine's proposed function in the clearance of apoptotic cells that is also predominantly non-inflammatory. Fractalkine is important in plaque stability and repair during atherosclerosis (Lucas, 2003). This paper has shown that the fractalkine released by the smooth muscle cells in atherosclerotic plaques helps to stabilise plaques and prevents rupture and subsequent thrombosis. The smooth muscle cells in the plaque secrete fractalkine that aids repair. Disappointingly, the fractalkine knockout mice and the CX3CR1 knockout mice appeared to have no obvious phenotype or disease (Jung, 2000; Cook, 2001). However, defects emerged when these mice were crossed with other mice. The Apo3 transgenic mouse has an accelerated process of atherosclerosis, and dramatic reductions in the size of plaques was seen when this mouse was crossed with the fractalkine receptor knockout mouse (Combadiere, 2003). The brain's

macrophages, the microglial cells can up-regulate fractalkine receptor in response to IL-10 a chemokine that is found in abundance in BL (Ogden, 2005; Kremlev, 2005). It is possible that the IL-10 environment in BL similarly up-regulates CX3CR1 expression of tumour-associated macrophages. When IL-10 was added to the microglial-like cell-line "HAPI" it overcame the effect of LPS and increased expression of CX3CR1. At the same time as promoting fractalkine-receptor expression, IL-10 reduced TNFα and CCL5 (RANTES) production, but neither IL-10 nor LPS alone affected fractalkine expression (Kremlev, 2005).

Geissmann's group showed that monocytes that expressed high levels of CX3CR1 trafficked away from a septic peritoneum and migrated into the lung. Generally, the lung is a non-inflammatory environment (Geissmann, 2003). Alveolar macrophages can deal with large amounts of inhaled LPS daily, and cope efficiently with pathogens without provoking damaging inflammation that might prevent gas exchange. More recently, a specialised, myeloid DC that expresses CX3CR1 has been described in the lamina propria of the murine intestine (Niess, 2005). This DC puts out dendrites into the gut lumen where it samples and phagocytoses both commensal and pathogenic bacteria. In the absence of the fractalkine receptor, these DCs did not put out dendrites and stopped sampling the intestine. The mice lacking the fractalkine receptor were susceptible to challenge by an invasive *S.typhimurium* and an additional phagocyte was recruited to deal with the infection. Their role in defence against entero-invasive bacteria seems to be in part, an early warning of *S.typhimurium*, and part tolerance to commensal bacteria.

Undoubtedly, fractalkine's role may change in different organs and whether it has pro or anti-inflammatory actions is influenced by the local tissue microenvironment. Fractalkine may have an anti-inflammatory role in tissues rich in IL-10 such as Burkitt's Lymphoma and perhaps in mouse strains such as the BALB/c (Ogden, 2005; Mills, 2000; Kremlev, 2005). When IL-10 is constitutively expressed by macrophages, under the control of CD68, they become "alternatively activated," however, it remains to be seen whether fractalkine receptor is a marker of the M2-macrophage (Lang, 2002). There is evidence that in the brain apoptotic cells secrete fractalkine after treatment with glutamate. The released fractalkine may guide

microglial (macrophage-like) cells towards the apoptotic cells in the brain (Maciejewski-Lenoir, 1999; Tong, 2000; Erichsen, 2003).

6.4 Fractalkine in Burkitt's lymphoma

Tumour-associated macrophages (TAM) have been described as "benign" in standard pathology textbooks (Cotran, 1989). TAM may influence the pathogenesis of Burkitt's (and related) lymphomas by multiple means, including helping the tumour to avoid immune detection as has been suggested for other malignancies (Mantovani, 2002). Results from Chapter 4 showed that apoptotic tumour cells recruited monocytes and macrophages *in vitro*. Once arrested in the tumour microenvironment, these cells may acquire an "alternatively-activated" phenotype through the engulfment of apoptotic cells. The release of TGF-β and IL-10 after the phagocytosis of apoptotic cells may aid tumour growth and allow evasion of anti-tumour immunity (Savill, 2000; Ogden, 2004).

Macrophages are the predominant infiltrating cell in Burkitt's lymphoma and may represent the tingible body macrophages seen in healthy germinal centres. BL tumours may trap monocytes and macrophages and subvert them into a non-inflammatory role to prevent them presenting tumour antigens. The tumour microenvironment, full of apoptotic cells and rich in IL-10 and may lead to the emergence of so-called "alternatively activated" macrophages.

In the transmigration assay, the apoptotic cells were artificially separated from the macrophages by the semi-porous filter. In a preliminary experiment, macrophages and BL-cells were reconstituted in an *in vivo* model of Burkitt's lymphoma. The BL2 Burkitt's lymphoma cell line was injected subcutaneously into SCID mice. In the absence of adaptive immunity, these mice developed tumours that had the characteristic "starry sky" appearance of the Burkitt's tumour seen in humans. Because F4/80-positive mouse macrophages were recruited into the human tumours this experiment showed that macrophage migration to BL-cells was conserved between the mouse and humans *in vivo* as had been seen *in vitro*. Next the SCID model will be used to test whether macrophage recruitment into BL is apoptosis-dependent. Further experiments using the CX3CR1-/- mouse are planned to see whether fractalkine is important in macrophage chemotaxis to apoptotic BL-

cells in the mouse. Immunohistochemistry of human Burkitt's lymphoma has shown that the macrophages infiltrating the tumours do indeed express the fractalkine receptor (CX3CR1). This preliminary observation requires further work to assess the importance of fractalkine in the pathogenesis of Burkitt's lymphoma *in vivo*.

6.5 Where are the cytotoxic cells in Burkitt's lymphoma?

Macrophages are the predominant cell infiltrating BL. In stark contrast to monocytes and macrophages, neutrophils failed to migrate to BL-cells despite migrating effectively towards 10^{-8}M fMLP *in vitro*. The negative levels of neutrophil migration towards both live and apoptotic Mutu BL-cells suggested that Mutu BL-cells release factor(s) that could inhibit neutrophil migration, independently of apoptosis. Neutrophils showed no evidence of degranulation in these experiments and >90% of cells were viable when viewed under the microscope. Apoptotic Mutu BL-cells seemed capable of actively preventing neutrophil transmigration, presumably through the release of soluble inhibitory-factors. Failure of neutrophil chemotaxis to Mutu BL-cells was independent of apoptosis, since Mutu $_{bcl\text{-}2,}$ (that had negligible apoptosis) and Mutu $_{UV,}$ (having large numbers of apoptotic cells) both inhibited chemotaxis. The observation that Mutu BL-cells were not chemoattractive to neutrophils reflects the histological picture where there is a paucity of polymorphonuclear cells infiltrating the Burkitt's tumours Figure 4.1a. Neutrophils do not highly express the fractalkine receptor CX3CR1 and this may be one explanation for their absence (Beck, 2003). However, neutrophils do express G2A, the receptor for LPC, another chemoattractant molecule released by apoptotic cells (Yang, 2005). Neutrophils also have the receptor for another chemoattractive molecule, S19. Apoptotic HL-60-cells release S19 that preferentially attracts monocytes but not neutrophils, despite the fact that both cells express its receptor, C5a. The lack of a neutrophil migration to S19 may be due to changes in the structure of S19 after contact with neutrophil proteases. This may turn S19 from a agonist into an antagonist (Nishiura, 1996).

Fractalkine receptor is expressed on natural killer cells and some CD8+ cytotoxic lymphocytes, both cells with the potential to destroy tumour cells like BL

(Guo, 2003; Morita, 2004; Yoneda, 2000; Nishimura, 2002; Campbell, 2001). There are no studies published to date describing NK cells in Burkitt's lymphoma. This is surprising, because NK-cells express high levels of the fractalkine receptor (CX3CR1) and have an important role in anti-tumour immunity and graft rejection. It has been shown that anti-fractalkine antibodies block NK-cell infiltration and prolong cardiac graft survival in rats (Haskell, 2001). Furthermore, Christophe Combadiere's group showed that transfection of a mouse embryo-cell line (E20) with fractalkine induced NK cell-infiltration and reduced tumour size (Lavergne, 2003). However, CD56+ NK cells are not a characteristic feature of Burkitt's tumours (Bridget Wilson personal communication). Are NK cells excluded from the tumour in the same way as neutrophils are repelled? NK cells, like monocytes and neutrophils, express the receptor G2A and should respond to LPC released by apoptotic cells (Lauber, 2003). There is evidence to suggest that NK cells become compartmentalised in the circulation where phospholipids like LPC are abundant. Resting NK cells may be held up in the circulation unless further inflammation triggers exudation of serum lipoproteins like LPC (Maghazachi, 2005).

6.6 Lysophosphatidylcholine

The finding that the chemoattractant molecule lysophosphatidylcholine (LPC) was released by apoptotic cells, was published during the course of these studies (Lauber, 2003). It is unlikely that fractalkine is the only chemoattractant molecule released by cells during apoptosis, and other chemoattractant molecules like LPC will also be important. LPC's major role may turn out to be more than chemotactic (Fadok, 2003; Ravichandran, 2003). There is some debate about whether LPC is pro or anti-inflammatory, however, recent evidence points to a non-inflammatory role. LPC was shown to antagonise its receptor G2A, and, when injected into mice, LPC prevented septic shock (Murkami, 2004; Wang, 2004). The mechanism might be caused by LPC compartmentalising monocytes in the circulation in the same way as it holds onto NK cells (Maghazachi, 2005). In this way, LPC would prevent monocyte migration into inflammed tissues thus stopping TNFα-release and septic shock (Beutler, 2001).

In serum, 99% of LPC is bound to albumin. Critically, all experiments in the Lauber paper, showing that LPC was chemotactic to macrophages, were done in the presence of serum (Lauber, 2003). LPC is found at such a high concentration in blood that it is difficult to imagine how a gradient would be formed between the circulation and the tissues that would allow for monocyte chemotaxis to an apoptotic cell. Perhaps there is a role for other chemokines, like fractalkine, in drawing monocytes out from the circulation towards apoptotic cells. The experiments presented here were all performed in the absence of serum. However, in common with the previous work, apoptotic cells were more prominently chemotactic for macrophages compared to monocytes. This may be because monocytes express less fractalkine receptor (CX3CR1) than macrophages. It is likely that the release of fractalkine by apoptotic cells is a local signal that aids the navigation of macrophages within tissues, rather than the movement of monocytes out of the circulation.

Any chemokine produced by an apoptotic cells should be released early on in the cell death programme in order to ensure swift clearance. This would allow time for phagocytes to respond to the chemotactic gradient generated by the dying cell. Critically, LPC, and fractalkine appear to be released early, when the cell's plasma membrane is intact. Release of fractalkine from the surface of viable cells is a complex, dynamic process and is tightly controlled by two metalloproteinase enzymes ADAM 10 and ADAM 17 (TACE).

ADAM 10 releases fractalkine constitutively and ADAM 17 (TACE) can be induced by inflammatory cytokines and phorbal esters (Garton, 2001; Hundhausen, 2003; Tsou, 2001). Recently, a novel pathway for recycling membrane-bound fractalkine has been described where intracellular fractalkine is stored in juxtanuclear vesicles (Liu, 2005). This represents an additional tier in the tight control of fractalkine release from cells, and may hint at the importance of the bioavailability of fractalkine. Like most chemokines, fractalkine's release is controlled at the level of transcription and translation, but the additional recycling pathway does not rely in de novo synthesis. Potentially, this means that a burst of preformed fractalkine could be rapidly mobilised early on during apoptosis. These experiments show that macrophage migration to apoptotic BL-cells occurs early after the induction of apoptosis and future experiments that measure the release of fractalkine are planned.

A powerful argument for the importance of LPC in apoptotic cell engulfment is the direct link between the release of LPC and the execution of apoptosis. The release of LPC was not specific for any particular cell type, but required the apoptotic enzyme, caspase 3 (Lauber, 2003). Similarly, the chemoattractive molecule S19, was induced during apoptosis (Nishiura, 1996). Lauber showed that the breast carcinoma cell-line MCF7 that has no caspase 3 did not attract monocytes but after transfection with caspase 3, apoptotic cells attracted monocytes. Although MCF7 lack caspase 3, they still pass through many of the morphological phases of apoptosis, they can flip phosphatidylserine and are efficiently phagocytosed by macrophages (Turner, 2003). However, in the absence of caspase 3, dying MCF7 cells may not be able to release LPC. Caspases are pivotal enzymes in a protease cascade that triggers apoptosis. The large number of bands seen by western blotting of fractalkine from apoptotic BL-cells suggests that proteolytic cleavage occurs during apoptosis. It will be interesting to discover whether the activity of TACE and ADAM 10 are influenced by caspases. The depletion of cholesterol from cell membranes can alter the activity of TACE and ADAM10 (Matthews, 2003). This suggests that a change in membrane fluidity, as occurs during apoptosis could affect cleavage of fractalkine as well as the release of membrane LPC.

LPC is linked to mitochondrial triggering of cell apoptosis through the *bcl-2* group member, *bid*; full-length *bid* binds to LPC and incorporates into the lipid bilayer of the mitochondria. This destabilises the mitochondria allowing the release of cytochrome C and the activation of caspase-9 (Goonesinghe, 2004). Redistribution of the membrane lipids like phosphatidylserine and lysophosphatidylcholine is not just important for apoptotic-cell phagocytosis but is also relevant to chemotaxis. Altering the LPC content of cells changes their membrane fluidity. The inverted-cone shape of LPC makes the membrane more convex and prevents its puncture by viral particles and conversely, stimulates pinocytosis of the membrane (Stiasny, 2004). Intercalation of LPC into the plasma membrane promotes blebbing and these blebs may contain MFG-E8 and autoantigens (Casciola-Rosen, 1994; Oshima, 2002) In addition, membrane blebs freed from the apoptotic cell surface may themselves be chemoattractive to macrophages. Germinal centre B cells isolated from tonsil undergo spontaneous apoptosis, releasing chemotactic molecules that are

concentrated in the blebs that pinch off from the apoptotic cell's membrane (Segundo, 1999). It may prove to be that fractalkine is concentrated in blebs that were chemoattractive to macrophages. Certainly, the particulate pattern of fractalkine surface-expression on apoptotic cells seen by confocal microscopy may represent a concentration of the chemokine in membrane blebs.

6.7 Fractalkine and MFG-E8

Rather than being recruited from circulating monocytes, it is possible that the TAMs that infiltrate BL are tissue macrophages that move in from nearby. Local rather than systemic chemokine networks may orchestrate which kind of cells nest amongst the tumour cells, allowing macrophage access but denying NK cells and neutrophils. Lymph node architecture is far from arbitrary and is controlled by cytokine members of the TNF-family like lymphotoxin and chemokines like BLC (CXCR5) (Mariathasan, 1995; Forster, 1996). In addition to these known chemokines, fractalkine and MFG-E8 might also have an influence. A role for MFG-E8 in germinal centre formation has just been described that may have implications for the study of BL.

With the identity of a receptor that directly binds to PS in doubt, more attention must be paid to opsonins such as MFG-E8 and C1q, that can link macrophages to apoptotic cells (Devitt, 2004; Borisenko, 2004). MFG-E8 binds oxidised PS on apoptotic cells and links (via its RGD motif) to integrin receptor $\alpha_3\beta_5$ or $\alpha_3\beta_2$. This work has shown that CD14 is up-regulated on macrophages that have migrated to apoptotic BL-cells. Might it be possible that MFG-E8 or other clearance receptors might similarly be up-regulated in readiness to phagocytose the dying target? This would neatly overcome the theoretical problem of fractalkine receptor desensitisation. As a macrophage moves up the chemokine concentration gradient fewer and fewer of its receptors are available and so it eventually stops moving at the site of highest chemokine concentration. This would prevent the macrophage using CX3CR1 to tether apoptotic cells displaying fractalkine. Currently, the evidence for MFG-E8 involvement in macrophage chemotaxis to apoptotic cells is indirect but very exciting.

New evidence has emerged that may link MFG-E8 to fractalkine-induced chemotaxis to apoptotic cells. MFG-E8 was identified to be significantly up-regulated in a micro-array of genes from macrophage-like microglia cells that had been treated with fractalkine (Leonardi-Essmann, 2005). If dying and damaged neurons release fractalkine that causes local macrophages to produce MFG-E8, couldn't the same thing be occurring in Burkitt's lymphoma? Early and preliminary experiments presented here show that TAM in BL express CX3CR1 but the presence of MFG-E8 has not been demonstrated in BL to date. The fact that tingible body macrophages express MFG-E8 (Hanayama, 2004), and that BL is a tumour that resembles a germinal centre B cell, makes the up-regulation of MFG-E8 by fractalkine more likely.

The MFG-E8 knockout mouse has a very interesting phenotype in that the MFG-E8-/- macrophages are able to tether apoptotic cells but do not phagocytose them, indicating that integrin signalling is important for the "tickling" stage of engulfment. Later on, MFG-E8-/- developed an autoimmune glomerulonephritis. Another extraordinary feature of this knockout was massive spleenomegaly caused by increased numbers of germinal centres. Despite being three times bigger than the wild type, the ration of B cells to T cells did not change in the knockout (Hanayama, 2004). This might suggest that MFG-E8 has a role in the regulation of germinal centre formation.

Conclusion

The search for a "come-get-me" chemokine began by looking at the phagocytic "tethering" receptor CD14, and ended with the discovery that fractalkine, a "chemokine on a stalk" was released by apoptotic BL-cells. If fractalkine behaves in BL in the same way as it does in the brain and *in vitro*, its release from apoptotic cells will prolong the life of the macrophages, perhaps by stimulating the PI3K/Akt pathway (Boehme, 2000; Shulby, 2004). Thus stimulated, the long-lived TAM might use MFG-E8 to efficiently engulf even more apoptotic cells, and secrete factors that stimulate tumour growth (Ogden, 2005). In this way, the persistence of apoptotic cells within BL maintains a non-inflammatory environment that allows the tumour to thrive, safe from immune detection.

The tumour suppressor gene p53, has been described as the most important gene in cancer (Greenblatt, 1994). As p53 accumulates in damaged cells, it prevents the defective DNA from replicating and may even drive the cell to commit suicide by apoptosis. The finding that fractalkine was a direct target of p53 is intriguing and fits with the idea that fractalkine release by apoptotic cells may not be limited to Burkitt's lymphoma, but is a ubiquitous process, going on in many different tissues (Shiraishi, 2000).

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Macrophage chemotaxis to apoptotic Burkitt's lymphoma cells *in vitro*: role of CD14 and CD36

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Abstract

In Burkitt's lymphoma (BL), apoptosis occurs at high frequency alongside uncontrolled proliferation. Macrophages infiltrate these tumours in large numbers and engage in the phagocytic clearance of apoptotic cells *in situ*. Here we tested the hypothesis that apoptosis of BL cells may provide a mechanism for recruitment of macrophages to these tumours. We show that monocytes and macrophages, but not neutrophils, preferentially migrated to apoptotic BL cells *in vitro*. Transfection of BL cells with the anti-apoptotic gene *bcl-2* both prevented apoptosis and abolished macrophage chemotaxis. Macrophage migration to BL populations correlated well with the number of apoptotic BL cells present (the Pearson correlation $r = 0.81$, $p < 0.0001$). Chemoattraction of murine macrophages to apoptotic human BL cells demonstrated that the mechanism was conserved across these species. In an attempt to identify the macrophage receptors involved in this process, we investigated whether CD14 and CD36, two receptors important in the phagocytic clearance of apoptotic cells, were also involved in the chemotactic macrophage response. We found that bone marrow-derived macrophages from $CD14^{-/-}$ and $CD36^{-/-}$ mice moved as well as wild-type macrophages in chemotaxis assays towards apoptotic BL cells. Migrating macrophages were found to be up-regulated in their expression of CD14, however, suggesting that, although this receptor does not appear to be required for 'sensing' apoptotic cells, it may be up-regulated on the surface of the migrating macrophage in readiness for apoptotic corpse clearance.
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Keywords: Macrophage, Burkitt's lymphoma, apoptosis, chemotaxis

Abbreviations: BL, Burkitt's lymphoma; BMDM, Bone marrow-derived macrophages; CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; CD, cluster of differentiation; DAPI, 4,6-diamidino-2-phenylindole; DC, Dendritic cell; EBV, Epstein-Barr virus; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocynate; fMLP, *N*-formylated-Methionine-Leucine-Phenylalanine; H&E, haematoxylin and eosin; LPS, lipopolysaccharide; mCSF, macrophage colony-stimulating factor; MM6, Mono-Mac 6; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PE, phycoerythrin; SEM, standard error of the mean; Supp, supplemented; TGF-β, Transforming-growth factor beta; UV, ultraviolet

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Introduction

Burkitt's lymphoma (BL) is a malignancy of germinal centre B-cell origin that is associated histologically with frequent apoptotic cells and infiltrating macrophages, which endow the tumour with its classical 'starry sky' appearance (Burkitt, 1958). The pyknotic nuclei are testament to the high rate of apoptosis that accompanies cell proliferation in the tumour and the macrophages are actively engulfing the numerous apoptotic cells they encounter. Apoptotic cells can be phagocytosed in many different tissues by neighbouring cells, including epithelial cells and fibroblasts, which act as 'amateur' phagocytes. However, when cell death occurs at high rates then macrophages, the 'professional' phagocytes, come into play and efficiently clear away apoptotic cells. By isolating the dying and dead cells from their environment, such clearance is not only a damage limitation measure, but also appears to actively suppress inflammatory and immune responses (Savill et al., 2002).

While much has been learned in recent years of the mechanisms by which apoptotic cells are recognised and engulfed by macrophages, little is yet known of the processes underlying the 'sensing' of apoptotic cells by phagocytes (Fadok, 1992). Recent studies have highlighted the importance of chemokine release during normal cell death and of how macrophages may sense and then move towards a dying cell in readiness to engulf the apoptotic corpse (Audran et al., 1996; Cocca et al., 2002; Horino et al., 1998; Kao et al., 1994; Lauber et al., 2003; Segundo et al., 1999). Faulty macrophage chemotaxis towards apoptotic cells might delay clearance and result in unwanted inflammation and a predisposition to autoimmune diseases like systemic lupus erythematosus (Herrmann, 1998; Taylor, 2000).

Chemotaxis is the directional movement of a cell up a chemokine gradient and is important in a number of biological processes including leukocyte recruitment to sites of inflammation, and wound healing (reviewed in Zlotnik and Yoshie, 2000). Here, we have examined the migration of macrophages to chemotactic molecules released from apoptotic BL cells. Migration and phagocytosis involve common membrane and cell shape changes and both processes are known to be regulated by common signalling pathways involving integrins and small G proteins (Aderem and Underhill, 1999; Hogg and Leitinger, 2001). Indeed, apoptotic-cell engulfment and cell migration in the nematode worm *Caenorhabditis elegans* have been shown to be governed by common genes that are conserved in flies and mammals (Wu and Horvitz, 1998). Given the mechanistic similarities between phagocytosis and chemotaxis, we reasoned that macrophage receptors involved in the recognition and engulfment of apoptotic cells might also function in the chemotactic response of macrophages to such cells. In this study, we have focussed on two receptors that have important roles in the clearance of apoptotic cells: the lipolysaccharide receptor, CD14 and the class B scavenger receptor, CD36.

Our previous work has shown that CD14 is not only involved in the clearance of apoptotic cells by human macrophages *in vitro* (Devitt et al., 1998) but also by murine macrophages *in vivo* (Devitt et al., submitted). Significantly, macrophages in multiple tissues of CD14-deficient mice show a defect in apoptotic-cell removal resulting in the persistence of apoptotic cells that are not associated with phagocytes *in situ*. One interpretation of this observation is that the absence of CD14 caused a chemotactic defect leading to a reduced efficiency of apoptotic-cell clearance. In this context, it is worth noting that neutrophils of the $CD14^{-/-}$ mouse have been shown to be tardy in migrating into an inflamed peritoneum (Yang et al., 2002).

Both CD14 and CD36 are known to bind lipids and in this respect may be appropriate candidate chemotactic receptors of macrophages that sense lipid moieties released from apoptotic cells. Of particular significance to this possibility is the recent observation that the lipid lysophosphatidylcholine is released from apoptotic cells and appears to function as a chemoattractant for macrophages (Lauber et al., 2003). CD36 is a well-characterised scavenger receptor for oxidised low-density lipoprotein that also recognises altered lipid on the surface of apoptotic cells (Ren et al., 1995; Savill et al., 1992). CD14, as well as binding glycolipids, also binds phospholipids, a function shared with CD36 (Sambrano and Steinberg, 1995; Yu and Wright, 1997). Here we use macrophages from $CD14^{-/-}$ and $CD36^{-/-}$ mice to investigate the possible role of these receptors in chemotaxis of macrophages towards apoptotic BL cells *in vitro*.

Materials and methods

Cells

The BL line, Mutu, and stable *bcl-2*-transfectant (Mutu $_{bcl\text{-}2}$) were obtained and cultured as described (Milner et al., 1992). Human peripheral blood was obtained from normal volunteers and buffy coats from the Scottish National Blood Transfusion Service. Ethical approval was obtained from the Lothian Research Ethics Committee. Human BL tumour biopsy was a gift from Katrina Wood, University of Newcastle-upon-Tyne. BALB/c wild-type mice and $CD14^{-/-}$ animals were purchased from B&K Universal (Hull, UK) and provided by Sanna Goyert, North Shore, New York, respectively. C57BL/6 wild type and $CD36^{-/-}$ mice were a gift from John Savill, Edinburgh University. The UK Government Home Office approved all animal

housing and procedures. Bone marrow derived macrophages (BMDM) and peritoneal macrophages were prepared from 8 to 12 week-old mice. Cell viability was assessed by trypan blue dye exclusion and purity by cytospin preparations of cells and by immunostaining and flow cytometry.

Macrophage culture

Mouse macrophages were cultured as previously described (Johnson et al., 1983; Ren et al., 2001; Savill et al., 1992). Briefly, for mouse BMDM, femurs were removed, cleaned and the marrow flushed with DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, ($DMEM_{supp}$)(Gibco, Paisley, UK) 10% heat inactivated FBS and 10% conditioned supernatant from L929 cells as a source of M-CSF (Labtech International, UK) (Hume and Gordon, 1983).

Peripheral blood mononuclear cells (PBMC) and neutrophils were isolated from the blood of normal donors by separation over a Percoll gradient (Amersham Pharmacia Biotech, UK). Neutrophils were washed in PBS and used immediately. Mononuclear cells were plated at 5×10^6 cells/ml in 6-well tissue culture plates (Costar). Lymphocytes were removed by washing after 1 h and adherent monocytes were either used immediately or matured into macrophages by culture for a further 8 days in $DMEM_{supp}$ and heat inactivated 10% autologous serum or human AB serum (Sigma).

Maintenance of cell lines

Mutu BL cells were cultured in suspension in RPMI supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin ($RPMI_{supp}$) and 10% serum supreme (Biowhittaker, MA). $Mutu_{bcl\text{-}2}$ were cultured with $RPMI_{supp}$ containing 2.5 mg/ml neomycin (Gibco Invitrogen, UK).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tumours were cut into 4 μm-thick sections and mounted onto Vectabond-coated glass slides (Vecta, CA). Briefly, slides were dewaxed in xylene, rehydrated and stained with haematoxylin and eosin (Shandon, UK). Slides were microwaved in antigen retrieval solution, blocked with biotin, avidin (Vecta, CA) and 5% normal goat serum (Harlan Sera-Lab, UK). For immunohistochemistry, slides were incubated with a mouse monoclonal anti-human CD68 (Dako, Denmark) followed by a biotinylated goat anti-mouse antibody (Dako). Positive cells were visualised using a streptavidin-HRP colour reaction with DAB (Dako) (Vecta, UK). Sections were counter-stained with haematoxylin (Shandon UK).

Induction of apoptosis

2×10^6 Cells were exposed to 100 mJ/cm^{-2} of UVB light and used 12 h later after washing in $RPMI_{supp}$ without serum.

Chemotaxis assay

Highly pure (>95%) neutrophils were used immediately in chemotaxis assays. Monocytes and macrophages were placed on ice for 5 min and harvested using a cell lifter (Costar), and then washed in $RPMI_{supp}$ without serum. 2×10^6/ml Mutu $_{bcl\text{-}2}$, Mutu or UV-treated Mutu were placed in 24 well plates. $RPMI_{supp}$ without serum was used as a negative control and 100 ng/ml human recombinant CCL5 (Peprotech) or 10^{-8} M fMLP (*N*-formylated-methionine–leucine–phenylalanine) (Sigma) as a positive control. Chemotaxis experiments were performed in polyvinyl uncoated transwells (Costar, 5 μm pores) using a modification of Boyden's technique (Barleon et al., 1996). Macrophages (10^5) were placed in 100 μl of $RPMI_{supp}$ in the top of a transwell and allowed to transmigrate to the bottom well at 37°C, 5% CO_2 for either 1 h (neutrophils) or 4 h (monocytes and macrophages). Control wells containing BL that had no contact with macrophages were set up in parallel to assess the amount of apoptosis at the end of the assay. Non migrated cells were removed from the upper well and the upper membrane was wiped. Filters were fixed in methanol and stained with Dif-Quik II (Dade, Germany). The number of cells that had migrated was counted in ten random high-power fields (×400 magnification) using an inverted microscope. All samples were prepared in duplicate and experiments were repeated a minimum of three times. The mean number of cells counted in ten high power fields was calculated and significance was measured using the Student's *t*-test. All data were graphed and analysed using Graph Pad Prism software version 3.02.

Fluorescence labelling and flow cytometry

All fluorescence labelling was carried out in the dark on ice. To measure apoptosis, cells were washed and maintained in calcium-containing buffer and labelled with annexin V and propidium iodide (Bender Med Systems, USA). CD14 expression was measured on transmigrated macrophages using a phycoerythrin-labelled (PE) monoclonal mouse anti-human CD14 and a PE-IgG2a isotype control (Dako, Denmark). All cells were analysed on a Coulter EPICS flow cytometer. Light scatter properties were used to identify zones

containing viable or apoptotic BL cells as described (Dive et al., 1992). For every experiment, the flow cytometric data was confirmed by analysis of nuclear morphology by staining with 4,6-diamidino-2-phenylindole (DAPI) (Sigma,UK). The percentage of cells with apoptotic morphology matched the number of cells falling in the 'apoptotic zone' by flow cytometry (data not shown).

Results

Macrophages phagocytose apoptotic cells in BL

BL is a small non-cleaved B cell tumour that contains many macrophages diffusely distributed amongst the "monotonous sea" of tumour cells. H&E staining (Fig. 1A) shows apoptotic lymphocytes with typical shrunken pyknotic nuclei (marked by an asterisk) that are more intensely stained than neighbouring live cells. The macrophages have large amounts of pale-staining cytoplasm that stand out clearly from the surrounding dark tumour nuclei. This creates the characteristic "starry sky" appearance of BL seen at low power. The cytoplasm of the macrophages is engorged with apoptotic bodies (marked with an arrow), indicating that macrophages are actively phagocytosing apoptotic cells *in situ*. The high numbers of macrophages distributed throughout the tumour are striking when the cells are labelled with anti-CD68, a macrophage marker (Fig. 1B).

Mutu BL cells expressing *bcl-2* are resistant to apoptosis

As cells undergo apoptosis they shrink in size and become more granular as nuclear chromatin clumps together forming bleb-like structures. These typical shape changes mean that dying cells can be clearly discriminated by flow cytometry (Dive et al., 1992). A representative plot is shown in Fig. 2. After a four-hour transmigration assay BL were removed from the lower chamber of the 24-well plate and the number of apoptotic cells was assessed using FACS. A typical forward versus side scatter plot of Mutu cells observed at the end of a transmigration assay is shown. When transfected with *bcl-2*, only 8.6% of Mutu $_{bcl\text{-}2}$ cells fell into the dead zone (Fig. 2A). Even 12 h post UV-treatment, Mutu transfected with *bcl-2* remained resistant to apoptosis and as little as 15% of cells entered the dead zone (data not shown). Since untreated Mutu BL cells undergo spontaneous apoptosis in culture, 27.5% of cells were apoptotic by the end of the experiment (Fig. 2B). However, 97% of UV-induced Mutu cells were apoptotic 12 h post-UV treatment (Fig. 2C).

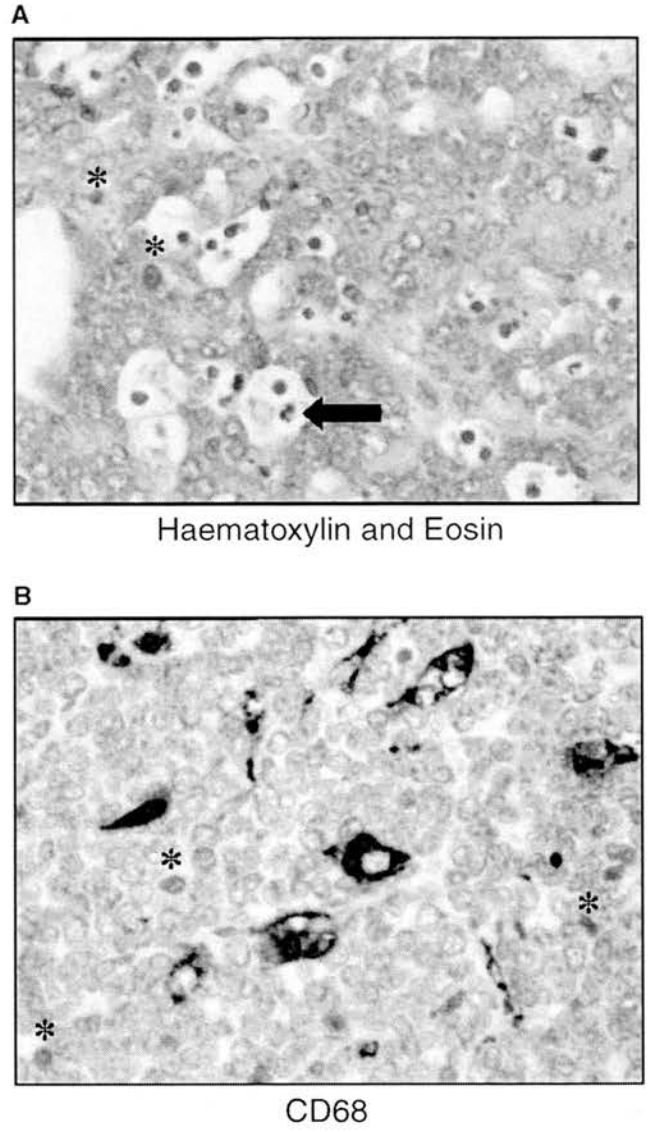


Fig. 1. Immunohistochemistry of BL: (A) High numbers of apoptotic cells (*) with condensed nuclei were seen in the tumour. Pale-staining tumour-associated macrophages were distributed in a typical "starry sky" pattern. Macrophages contained phagocytosed apoptotic bodies in their cytoplasm (arrow). (B) Positive CD68 staining (shown in black) distinguished macrophages from surrounding tumour cells.

Chemotaxis of human monocytes and macrophages to apoptotic Mutu BL cells

In transmigration assays, we saw that monocytes and 8-day old macrophages preferentially migrated to apoptotic Mutu cells (Fig. 3A and B). 100 ng/ml CCL5 (human RANTES) in RPMI was used as a positive control and media alone as the negative control in these experiments. Checkerboard experiments (including chemoattractant in either the upper or lower wells) revealed

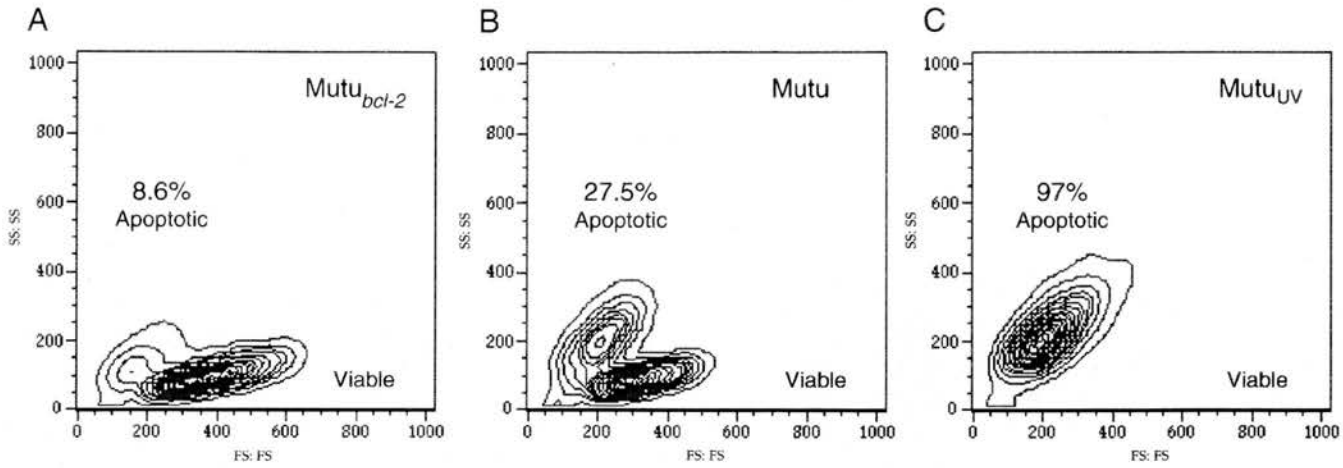


Fig. 2. Light scatter flow cytometry plots of Mutu BL populations showing 'viable' and 'apoptotic' zones according to Dive et al., 1992. Percentages refer to the number of apoptotic cells falling in the 'apoptotic' zone: (A) Mutu BL cells transfected with *bcl-2* were resistant to apoptosis and only 8.6% were apoptotic. (B) 27.5% of Mutu BL cells underwent spontaneous apoptosis in culture. (C) 97% of Mutu cells were apoptotic 16 h following UV-treatment. FS: forward scatter. SS: side scatter.

that macrophage migration was true chemotaxis and not simply chemokinesis (data not shown). Monocyte and macrophage chemotaxis to UV-treated Mutu BL cells was significantly higher than to Mutu $_{bcl-2}$ (Student's *t*-test $^{***}p<0.0001$), macrophage chemotaxis towards the *bcl-2*-expressing BL cells being virtually abolished. Macrophage migration to untreated Mutu depended on the number of (spontaneously) apoptotic cells present and was always higher than Mutu $_{bcl-2}$. Macrophages migrated preferentially to UV-treated Mutu that were over 90% apoptotic. The monocytic cell line Mono Mac 6 (MM6) also transmigrated to apoptotic BL cells (data not shown) in agreement with published results using this cell line in chemotaxis assays in other systems (Lauber et al., 2003; Ziegler-Heitbrock et al., 1988).

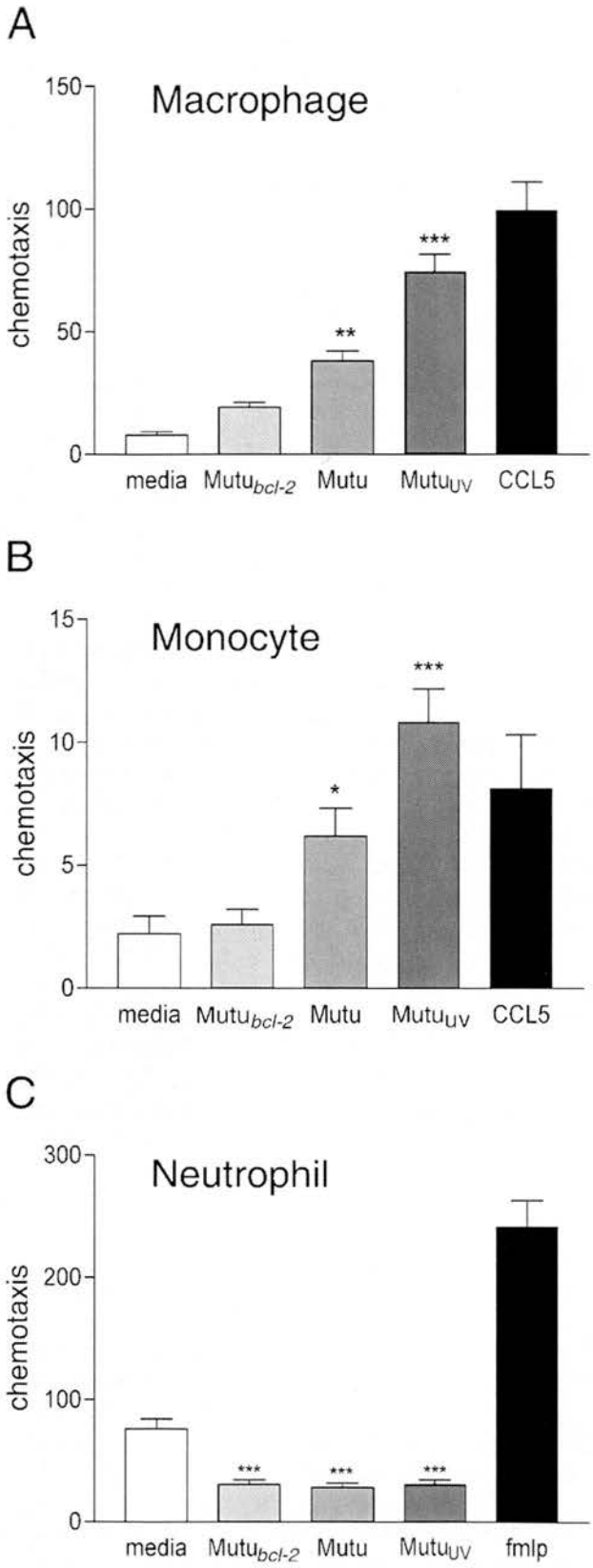
In stark contrast to monocytes and macrophages, neutrophils failed to migrate to BL cells despite migrating effectively towards 10^{-8} M fMLP (Fig. 3C). In these experiments, >90% of the neutrophils were viable (as determined by tryphan blue exclusion) at the end of the short (1 h) migration period. Cytospin preparations of neutrophils also showed no evidence of degranulation. The extremely low levels of migration of neutrophils towards all three BL preparations (lower than to media alone, Fig. 3C) suggested that BL cells release factor(s) that can inhibit neutrophil migration, independently of apoptosis.

Macrophage chemotaxis correlates with the number of apoptotic Mutu BL cells

Because the percentage of apoptotic cells varied slightly between individual experiments we carefully measured apoptosis by FACS using FITC-labelled Annexin V. Annexin V binds exposed phosphatidylserine on the outer leaflet of the plasma membrane of apoptotic cells. We next compared the percentage of Annexin V-positive cells with the chemotactic index taken from 23 experiments altogether. The chemotactic index refers to the fold increase in the number of transmigrated macrophages above the negative (media) control (Fig. 4). Here, Mutu $_{bcl-2}$ had low levels of apoptotic cells, a low number of Annexin V positive cells and stimulated very little macrophage transmigration. Mutu$_{UV}$ had a high percentage of Annexin V positive cells and also a high number of transmigrated macrophages. The correlation between the percentage of cells binding Annexin V and macrophage chemotaxis, was striking ($n=10$, Pearson's $r=0.81$, $^{***}p<0.0001$). These results show that dying Mutu cells release one or more chemotactic molecules that specifically attract monocytes and macrophages.

CD14$^{-/-}$ and CD36$^{-/-}$ macrophages have no chemotaxis defect

When we phenotyped the human macrophages that had transmigrated in response to apoptotic Mutu BL cells we noted that they expressed higher levels of the phagocytic receptor CD14 compared to input macrophages (Fig. 5A). We adapted our transmigration assay to use mouse bone marrow-derived macrophages. Using 10^{-8} M fMLP as a positive control, we saw that mouse BMDM migration to human apoptotic Mutu BL was a conserved phenomenon between these species. Mouse BMDM also preferentially migrated to apoptotic Mutu BL cells in the same way that human macrophages had (data not shown). We then examined macrophages



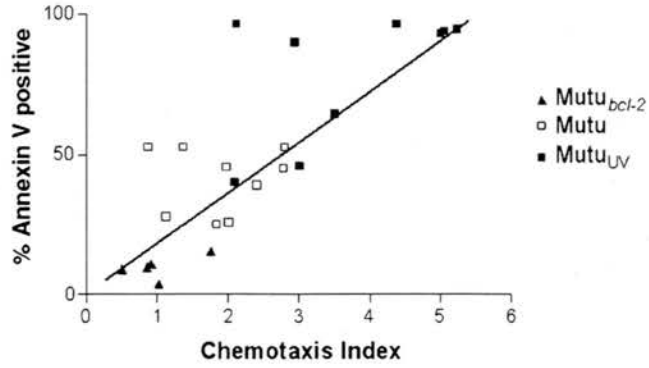


Fig. 4. Macrophage migration to Mutu BL cells is correlated to the number of apoptotic cells. Apoptosis was measured as the percentage of Mutu binding FITC-labelled Annexin V as analysed by flow cytometry. The chemotaxis index is the fold increase in the number of transmigrated macrophages compared to media (negative) control. Results from 23 separate experiments are plotted. The Pearson correlation $r = 0.81$, Student's t-test $^{***}p < 0.0001$.

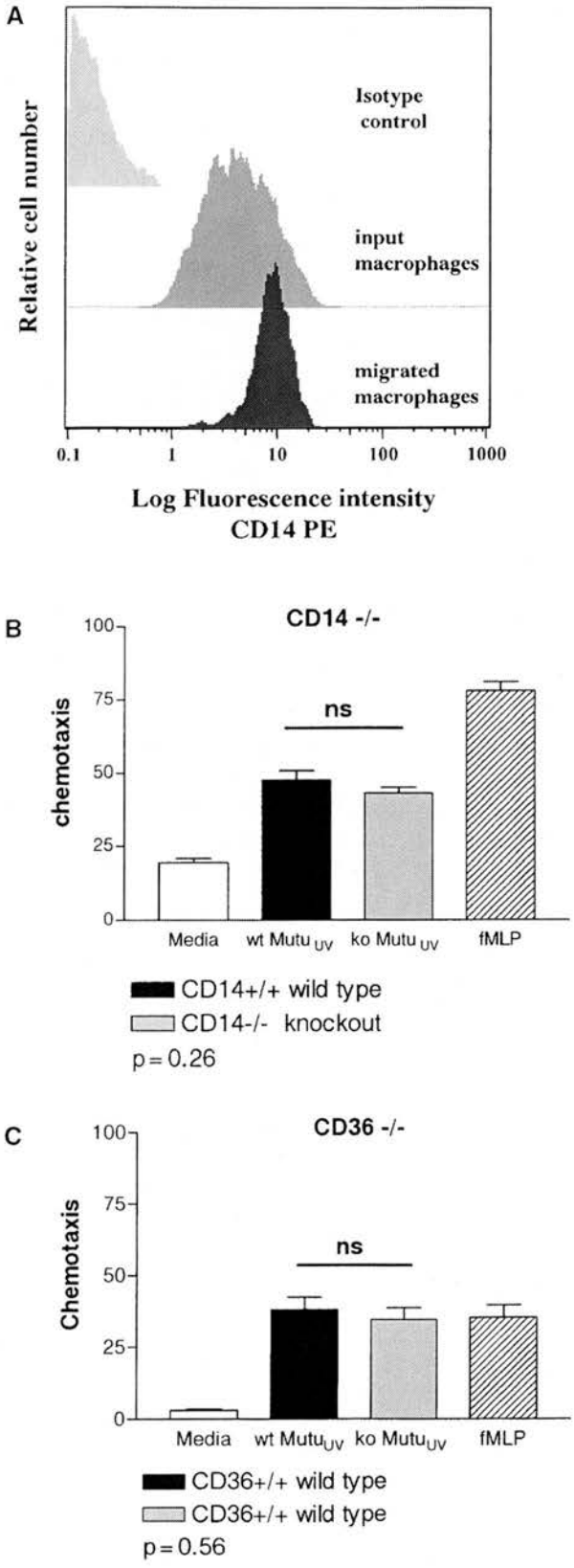
deficient in the phagocytic receptors CD14 and CD36. BMDM from CD14$^{-/-}$ mice had no defect in chemotaxis to apoptotic BL cells (Fig. 5B). Similarly, BMDM from the CD36$^{-/-}$ mouse showed no defect in chemotaxis to apoptotic BL cells compared to wild-type macrophages (Fig. 5C). Although important for apoptotic-cell clearance, these results indicate that CD14 and CD36 are not necessary for macrophage chemotaxis to apoptotic cells.

Discussion

The results presented here demonstrate monocyte and macrophage chemotaxis to apoptotic BL cells *in vitro* for the first time. Currently, we are investigating whether apoptosis may also be a mechanism for recruiting monocytes and macrophages to lymphomas *in vivo*.

Tumour-associated macrophages have been described as "benign" in standard pathology textbooks (Cotran

Fig. 3. Mononuclear phagocyte chemotaxis to apoptotic Mutu BL cells. Apoptosis was induced by UV-light. Chemotaxis was measured as the mean number of transmigrated cells counted in ten high power fields: (A) Macrophage chemotaxis to apoptotic Mutu BL cells. (B) Monocyte chemotaxis to apoptotic Mutu BL cells. For A and B media alone was the negative control and 100 ng/ml CCL5 was the positive control. (C) Absence of neutrophil chemotaxis to apoptotic Mutu BL cells. Media alone was the negative control and 10^{-8} M fMLP was the positive control. One representative result from at least three separate experiments is shown. Data represent means ± SEM; Student's t-test $^{***}p < 0.0001$, $^{**}p < 0.005$, $^{*}p < 0.02$.



et al., 1989) but they may influence the pathogenesis of Burkitt's (and related) lymphomas by multiple means including helping the tumour to avoid immune detection as has been suggested for other malignancies (Mantovani et al., 2002). Our data shows that apoptotic tumour cells can recruit monocytes and macrophages *in vitro*. Once arrested in the tumour microenvironment, these cells are likely to acquire an 'alternatively activated' phenotype and engage in the engulfment of apoptotic cells. The resulting anti-inflammatory effects, such as the release of TGF-β (Savill and Fadok, 2000), may aid tumour growth through evasion of adaptive anti-tumour immunity.

BL were not chemoattractive to neutrophils and appeared to be capable of actively preventing neutrophil transmigration, presumably through the release of soluble inhibitory-factors. Failure of neutrophil chemotaxis to BL cells appeared to be independent of apoptosis, since, $Mutu_{bcl\text{-}2}$, with negligible apoptosis and $Mutu_{UV}$, containing large numbers of apoptotic cells, both inhibited neutrophil chemotaxis. This was reflected in the histological picture seen in Fig. 1, which showed a paucity of polymorphonuclear cells infiltrating BL. This observation requires further work although one prediction might be that BL cells fail to secrete any CXCL chemokines expressing the ELR (Glutamate–Leucine–Arginine) tripeptide sequence, a chemokine group that is known to attract neutrophils.

Infection of cells with Epstein-Barr virus (EBV) can have profound immunomodulatory effects both *in vitro* and *in vivo*. EBV-positive Hodgkin's lymphomas have increased secretion of CCL5 and CXCL10 (IP-10), whereas mononuclear cells from EBV-infected individuals produce no CCL3 (MIP-1α) even after stimulation with LPS (Jabs et al., 2002; Teruya-Feldstein et al., 2000). Since Mutu is an EBV positive BL line, we repeated our experiments in an EBV negative BL line, BL2 (data not shown). Again, we demonstrated that macrophages migrated preferentially to apoptotic BL2

Fig. 5. (A) Human macrophages that had migrated to apoptotic cells expressed high levels of CD14. Flow cytometric histograms are shown of immunolabelled CD14-PE input versus migrated macrophages. (B) Mouse BMDM from the $CD14^{-/-}$ mice had no defect in chemotaxis to apoptotic Mutu BL cells. Media alone was the negative control and 10^{-8} M fMLP was the positive control. Chemotaxis was measured as the mean number of transmigrated cells counted in ten high-power fields. One representative result from at least three separate experiments is shown. Data shown are means $\pm$ SEM; Student's *t*-test $p = 0.26$. (C) Mouse BMDM from the $CD36^{-/-}$ have no defect in chemotaxis to apoptotic Mutu BL cells. One representative result from at least four separate experiments is shown. Data shown are means $\pm$ SEM; Student's *t*-test $p = 0.56$.

cells. This experiment excluded the possibility that EBV itself had caused the macrophage chemotaxis.

We saw chemotaxis to both spontaneous and UV-induced apoptotic cells. This suggests that chemotaxis was to products released during the process of apoptosis and was not dependent on the (UV) stimulus that induced cell death. Macrophages were also able to migrate to apoptotic cells that had been induced by stimuli other than UV, like staurosporin and ionomycin (data not shown). Stable transfection of Mutu BL cells with *bcl-2* reduced the high level of spontaneous apoptosis in Mutu to low levels. It is conceivable that *bcl-2* acts by inducing chemokine expression by Mutu as has been shown with *bcl-xL* in macrophages (Lakics et al., 2000). However, even when Mutu $_{bcl\text{-}2}$ data is excluded, there remains a strong correlation between the number of apoptotic cells and chemotaxis (when UV-treated Mutu cells are compared to untransfected Mutu cells) (Pearson's $r = 0.73$, $^{***}p < 0.001$). BL that retain the features of the tumour biopsy cells have high levels of spontaneous apoptosis and so it proved impossible to obtain a 100% pure population of live Mutu without some contaminating apoptotic cells. Methods to remove apoptotic cells such as density gradient centrifugation, reduced the numbers of dead cells but were not as effective as *bcl-2* transfection. The question of whether *bcl-2* could inhibit chemotaxis in the current model through mechanisms other than inhibition of apoptosis deserves further attention.

Although CD14 and CD36, lipid-binding receptors of macrophages that are known to function in apoptotic cell clearance, are not members of the chemokine receptor family, the discovery of a lipid chemokine factor secreted from apoptotic cells (Lauber et al., 2003) led us to rationalise that these receptors may play a role in macrophage chemotaxis towards such cells. Integrin signalling is important in the firm adhesion of migrating cells to the endothelium during chemotaxis and macrophages combine CD36 together with the integrin $\alpha_V\beta_3$ in a complex to phagocytose apoptotic cells (Audran et al., 1996; Savill et al., 1992). CD14 is a glycophosphatidylinositol-linked receptor with no intracellular domain and so needs to cooperate with receptors like Toll-like receptor 4 and CD11b in order to signal, (Ingalls et al., 1998; Jiang et al., 2000). However, co-localisation of CD14 with a classical chemokine receptor has not been described to date. We observed that transmigrated macrophages expressed high levels of CD14 compared to the input population. This may represent either up-regulation of CD14 during chemotaxis, or the specific migration of the CD14-high macrophage subset towards the apoptotic cells. Study of human monocyte subsets showed that CD14-high monocytes expressed CCR2 and moved towards CCL1 (MCP-1) (Weber et al., 2000) and CD14-low monocytes failed to respond to this chemokine but moved preferentially to CX3CL1 (fractalkine) (Ancuta et al., 2003). Murine homologues of the CD14-high and low subsets have been described which also have distinct migratory properties (Geissmann et al., 2003). It is tempting to speculate that the CD14 on the migrated macrophages is up-regulated in readiness for efficient corpse recognition and removal.

Despite observing that transmigrated human macrophages expressed high levels of CD14, we saw no chemotactic defect in macrophages from $CD14^{-/-}$ mice. Although CD14 is a marker for monocytes with distinct migratory potentials, our results show that CD14 is not required for macrophage chemotaxis to apoptotic cells. *In vivo* studies of bacterial peritonitis have also shown that F4/80-positive monocytes in $CD14^{-/-}$ mice were recruited as efficiently as wild-type controls (Henneke et al., 2002). Similarly, macrophages from the $CD36^{-/-}$ mouse also had no chemotaxis defect, migrating equally well towards apoptotic BL cells as wild-type controls.

In conclusion, the *in vitro* results presented here demonstrate that apoptotic tumour cells have the potential to play a key role in recruiting the macrophages that constitute a substantial proportion of the cell mass in BL. If this proves to be the case *in vivo*, this will have important implications for the role of both the apoptotic tumour cell and the macrophage in BL pathogenesis. Although "eat-me" signals displayed by dying cells have been well described, apoptotic-cell "find me" signals are mostly unknown to date. Towards the end of its life, a dying cell may well release lipids to attract phagocytes but our results have shown that macrophage chemotaxis to an apoptotic cell does not require the 'phagocytic lipid receptors' CD14 and CD36.

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